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Department of Anatomy, School of Medicine
and
Department of Zoology, College of Science
Southern Illinois University
Carbondale, Il 62901

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DEDICATION

We wish to dedicate this volume to the late John Chapman Frye (1912-1982), formerly Chief of the Illinois State Geological Survey.

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It is my pleasure to acknowledge the kindness of many friends and colleagues, whose substantive suggestions and comments greatly improved the accuracy and completeness of this journal. A special note of appreciation to Dr. L. Richard Coulson for his willingness to tackle chores, his encouragement, and incredible endurance. To Dr. Harold M. Kaplan, my special gratitude is extended. His devoted attention to the preparation of this journal and his innate sense of style, taste, logic and his wise counsel have contributed immensely to the achievement of this volume. I am indebted to Ms. Peggy Stalcup, who shouldered the burden of the correspondence and endless details. I must express my most sincere thanks for the friendship, concern, and support we received over the years from Dr. Richard Moy, Dr. Robert Jackson, Dr. Jaime Estavillo, and Mr. John Thomas Williams of the Southern Illinois University School of Medicine. The cooperation and enthusiasm of the staff at Phillips Brothers, Inc. are sincerely appreciated.

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Anthony A. Paparo

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The Genital Tract of the Male Opossum, *Didelphis Marsupialis Virginiana*, and Other Marsupials.

Jan Martan
Department of Zoology
Southern Illinois University
Carbondale, Illinois 62901

In this paper an attempt has been made to collect and summarize available information on the male genital tract of *D. marsupialis*. When literature was available, information on other members of the family Didelphidae and other marsupials has been compared and conclusions drawn concerning the similarities and differences in structure and function of the male genital tract within and between the two groups. Any unifying concept which will emerge may serve as a base to distinguish between marsupials and eutherian mammals, and for speculation.

The prostate gland of *D. marsupialis* is a carrot-shaped structure which originates from urethal epithelium, and in the adult surrounds the proximal urethra (Moore 1941). It varies in length and diameter according to the age of the animal (Chase, 1939; Oudemans, 1892). It is narrow and straight in young males and enlarged, curved and even looped in adults. Histologically, the opossum prostate gland is of tubular variety. The secretory glandular tubules encircle the urethra and are embedded in the urethral stroma but do not penetrate the overlying smooth muscle layers. The ducts of the tubular glands open individually into the urethra (Chase, 1939). This type of prostate gland, in which glandular components do not penetrate the muscle layers of the urethra, is called a diffuse or disseminate prostate gland (Fig. 1). This arrangement contrasts with that in which parenchymal cells penetrate muscle layers and form a discrete glandular body outside the urethra (Price and Williams-Ashman, 1961). The opossum prostate gland is subdivided into 3 parts, each of which in the adult male has a characteristic diameter and color (Figs. 1, 2, 3, 4).

Chase (1939) designated the short, most craniad region which is light brown in color as prostate I, the medial region which has the greatest diameter and is pink in color as prostate II, and the most caudad gray colored region with the smallest diameter as prostate III. Following prostate III the urethral diameter marrows abruptly into the short membranous urethra extending to Cowper's glands, from which it broadens and continues as a cavernous urethra (Fig. 1).

Price and Williams-Ashman (1961) suggested that the 3 differently colored regions of the opossum prostate gland probably differed in structure and function as do the different lobes of prostates in eutherian mammals. Martan & Allen (1965) and Hruban *et al.* (1965) referred to these regions as prostatic segments I, II, and III. Histological studies of the opossum prostate by Chase (1939) showed each colored region of the gland to have a characteristic epithelium. The epithelial cells were: cuboidal to low columnar in segment I; columnar with apical granular material in segment II; and cuboidal with clear cytoplasm in segment III. The secretory tubules in segment I had narrow lumina and were separated from each other by distinct layers of connective tissue, while these tubules in segments II and III had broader lumina and were separated by narrow streaks of connective tissue.

Martan and Allen (1965), using various staining techniques and histochemical reactions, found great diversity in the cytological and cytochemical characteristics of cells from individual segments (Figs. 2, 3, 4). The distinctive feature of segment I was the large cytoplasmic globules derived by apocrine secretion and present in the lumina of the secretory tubules. The globules contained granules and vesicles, some of which showed positive reactions for phospholipids, polysaccharides, acid phosphatase (Fig. 21), aliesterase, nucleoside diphosphatase and 5′-nucleotidase. Similar reactions occurred in the cytoplasm of epithelial cells. The finding of an unusually high content of iron in the luminal globules and epithelial cells prompted Hruban *et al.* (1965) to suggest that the presence of iron gives segment I its brown color (Fig. 18).

Two types of columnar cells designated A and B cells (Martan and Allen 1965) characterized the secretory epithelium of tubules in segment II (Fig. 7). The cytoplasm of the taller A cells was filled with round granules which showed positive histochemical reactions for mucopolysaccharides. The cytoplasm of the less elongated B cells contained large numbers of fusiform granules which were strongly reactive with various histochemical tests for protein (Fig. 8). The cytoplasmic network around granules in both cells gave positive reactions for aliesterase, leucine amino peptidase, 5′-nucleotidase and nucleoside diphosphatase but reactions of both types of granules were negative. Tests for RNA were positive with a netlike distribution similar to that for enzymes. Secretory material found in the lumina of the glands and ducts of segment II gave positive reactions similar to those for the granules of type A and B cells. The presence of cytoplasmic granules was also reported by Ladman and Soper (1963).

The cuboidal cells lining the secretory tubules of segment III contained large globules of glycogen (Figs. 9, 10). This high content of glycogen (2g per 100g of wet tissue, Martan and Allen, 1965), was the characteristic feature of segment III. The large size of the glycogen mass in the cytoplasm of the cuboidal cells resulted in a peripheral distribution of cytoplasmic RNA, Golgi complex, and mitochondria. Phospholipids were localized in the apical portion of the epithelial cells. The lumina of the glands and ducts were filled with glycogen globules forming islands in an amorphous mass of phospholipids. This mass also gave a positive reaction for acid phosphatase (Fig. 6). In the cytoplasm only randomly distributed large granules were positive for this enzyme. Hardin (1965) also reported the presence of glycogen in segment III.

Hruban *et al.*, (1965) used electron microscopy to study cytological diversity of the opossum prostate gland. The granules in A and B cells of segment II, were both limited by a single membrane. The elongated granules in B cells had a fila-

mentous dense center, which in cross-sections appeared hexagonal. This center was surrounded by a less dense matrix. The granules from A cells were moderately electron dense and sometimes contained a small dense disc. The authors described another type of cell found in the outermost zone of segment II, which they called C cells (Fig. 5). The cytoplasmic granules of these cells were heterogeneous in nature and many had highly complex cylindrical structures inside the granules. The luminal surfaces of epithelial cells of all three segments, were covered by microvilli. Glandular lumina of segment III contained electron transparent globules of glycogen surrounded by masses of smooth membranes. Most membranes were aligned in parallel stacks. Electron microscopic studies showed ferritin-like particles in the apical parts of epithelial cells and the lumina of segment I.

In their cytochemical studies, Hruban *et al.*, (1965) reported positive reactions for thiamine pyrophosphatase, adenosine diphosphatase, inosine diphosphatase, and adenosine triphosphatase in the luminal content of all three segments. The secretory material in the lumina of segment III was also strongly positive for peroxidase. The results of their biochemical studies are summarized in Table 1.

Table 1. Biochemical studies on opossum prostate

Segment		I	II	III
Zinc	Micrograms per gram of wet tissue	15;63;13	18;40;17	19;57;38
Copper		6;12	2; 6	2; 8
Calcium		37;10	12; 6	13; 7
Magnesium		16;35	15;15	16;18
Iodine		6.1;1.6	0.8	2.0
Glycogen	mg/g	1.6	1.3	31.8
Glycogen synthetase without glucose-6-P	units/g	—	5.5	2.7
Glycogen synthetase with glucose-6-P	units/g	—	14	32
Catalase	units/g/sec	39;55	7; 7;14	615;1290
Uricase	units/g/min	0	trace	0
Lactate dehydrogenase	units/g/min	349	424	296

Values for metals and catalase are given on individual animals; each value represents the mean of two determinations.

(From Z. Hruban *et al.*, J. Exper. Zool., 160, 81-105, 1965, Alan R. Liss Inc.)

Chase (1939) studied the influence of castration on the opossum prostate gland by removing the entire scrota with their contents from adults. Generally, prostates of castrated males were lighter than those of controls, however, histological obser-

vations showed different responses by individual segments to castration. In all segments, after 90 days, the tubular glands had narrower diameters and their cells were cuboidal. The lumina of the glands were also narrower with little or no secretion. In segments I and II the lumen of the prostatic urethra was wider than in control animals, and segments II and III showed a noticeable increase in connective tissue in castrates. Chase stated that the greatest changes were in segment I where in some castrates the tubular glands were either disorganized or their glandular character lost. In the same study, of three adult male hemicastrates injected twice a day with gonadotrophins for 20 days, only the two receiving the higher doses showed increased weight of prostate glands when compared to normal controls.

A comparative study of cells in prostate gland of members of 3 genera of the family Didelphidae was done by Martan *et al.*, 1967a. (Unpub. Ms.). Seven four-eyed opossums (*Philander opossum*, Brisson), six murine opossums (*Marmosa* sp., Gray), and four woolly opossums (*Caluromys derbianus* Allen), all males, were obtained commercially. The reproductive maturity of the opossums was judged by the presence of spermatogenesis in the testes, curvature of the prostatic urethra, and distinct colors of individual prostatic segments.

The gross features of the prostate glands of these opossums were strikingly similar to those described in *Didelphis marsupialis virginiana*. Similarity was also observed on the microscopic level but to a different degree in various segments. The epithelial cells of secretory tubules in segment I were columnar with a distinct brush border. A strong positive reaction for glycogen was observed in the epithelial cells and lumina of segment I in all opossums. In addition to glycogen, the lumina also contained large cytoplasmic globules formed probably by a process of apocrine secretion. The glycogen masses in all opossums were often tinged with Alcian blue, suggesting the presence of mucopolysaccharides.

Histochemical tests for iron were positive only in the epithelial cells and lumina of the four-eyed opossums but to a lesser extent than in North American opossum. Granules reacting positively for iron were also PAS positive and diastase resistant. Hruban *et al.* (1965) suggested that the light brown color of segment I of the North American opossum was probably due to its high content of iron. In other species, where the reaction for iron was weak or negative, the color of the first segment was pale gray (Table 2).

The tall columnar epithelial cells of segment II in all opossums were filled with secretory granules. The granules differed in shape and size from species to species. However, their staining properties were similar. With the exception of the woolly opossum, A and B granules were localized in different types of epithelial cells. The B granules gave a strong positive reaction for protein: the staining reactions of A granules with PAS and Luxon Fast blue MBS techniques indicated the presence of polysaccharides and phospholipids. The epithelial cells in segment II of the woolly opposum were of one type with two types of oval granules demonstrable within the cytoplasm of most cells. Some granules had staining reactions characteristic of A cell granules of other opossums, while other granules gave reactions typical of B cell granules. The shorter cells, lining the ducts of segment II in all opossums, also contained granules typical for this segment. The granules were smaller, their staining reactions weaker and they were fewer in number. In the woolly opossum some of the duct cells contained glycogen. In all opossums the same granules as described in eipthelial cells were found also in the lumina of secretory tubules and ducts.

The third segment of all investigated species was rich in glycogen. Glycogen was found in the columnar epithelial cells lining the secretory tubules and in the cuboidal cells lining the ducts. The only exception was middle zone of the four-eyed opossum where the complex secretory products gave staining reactions for polysaccharides, proteins, and phospholipids.

The characteristic feature of all lumina in segment III, in all opossums, was the presence of amorphous masses in which granules and globules of glycogen were embedded. The masses gave a weak histochemical reaction for mucopolysaccharides, and in the murine and woolly opossums, for phospholipids as well. Biochemical studies (Table 3) demonstrated that the third segment in all investigated species, had catalase activity several times higher than that observed in the livers of these animals. In addition, segment I of the four-eyed opossum contained a high degree of catalase (Table 3). The prostatic catalase may be localized in the cells in the region of microvilli, which, in the North American opossum, showed high peroxidase activity (Hruban *et al.*, 1965). The differences in the relative sizes of comparable prostatic segments (Table 2) will probably be reflected in ejaculates and related to the metabolic requirements of ejaculated spermotozoa.

The genital tract of male *Marmosa robinsoni*, another species of the family Didelphidae, was described by Barnes (1977). The author stated that the prostate gland was unusually large (3.59% of the male body weight) in sexually mature males, had the same three distinctive segments as reported for other opossums, and there were two pairs of Cowper's glands.

Three North American male opossums *(Didelphis Marsupialis virginiana)* were reared in captivity and killed at the age of six months (Martan *et al.*, 1967a.). Their prostatic urethrae were not curved, indicating reproductive immaturity (Chase 1939). The prostrates were, however, already differentiated into three segments with distinct colors. Cytological staining reactions of all segments were similar to those of adult males (Hruban *et al.*, 1965; Martan and Allen, 1965). The cellular secretory activity in the young animals, however, was weak and unequally distributed. The lumina contained fewer secretory products. The secretory activity in all three segments appeared earlier on the ventral side of the prostate gland than on the dorsal side. In all segments, the cells close to the periphery of the gland matured later than cells in more central locations. Mitotic figures were absent in segment I, many were observed in segment II, and the greatest number occurred in segment III. The mitotic activity in the last two segments was greater at the periphery of the gland than in the middle. These observations suggest that segment I differentiated first and the third segment last. Due to the morphological similarity of prostates in all investigated opossums, this differential maturation of segments of prostatic glands in subadult males may be indicative of all Didelphidae.

Development of the prostate gland in pouch opossum males was studied by Chase (1939) and Moore (1941). Chase reported that the first appearance of prostate gland buds was noticed at 13 days, the first branching of these primordia at 28-30 days and the tree like solid cords, were characteristic of the prostate gland at 70 days. For similar stages, Moore listed 15, 40 and 100 days of development. Moore believed that in young male opossums, the fully developed functional prostate was achieved at approximately one year of age.

Prostate gland development of the pouch opossum was greatly stimulated and accelerated by androgen injections (Moore 1941), especially in the older males. In

Didelphidae, the female embryos do not have prostatic primordia at any stage of their genital tract development, yet the androgen injections induced prostate gland buds on day 16 in opossum females injected from the third day in the pouch (Moore 1941). Continued treatment of pouch females resulted in greater prostatic growth than that in the untreated males but lesser than that of similarly treated males of the same age.

Burns (1949) stated that introduction of androgen into pouch opossum females not only induced the prostate gland development but their urogenital sinus was changed into the male bilobed form. After androgen treatment was stopped there was no immediate regression of induced prostate glands in the females. Both authors agreed that estrogen injections were highly toxic to pouch opossum males. In the surviving young males there was no development of prostate glands. In older males where prostatic development already had started, the glands were modified by estrogen (Moore 1941). The male form of the urogenital sinus in estrogen treated males, was changed into that of the 5-lobed female form (Burns 1949). The failure of gonadotrophins to elicit acceleration of prostatic development in pouch opossum males younger than 70 days, caused Moore and Morgan (1943) to doubt that the testes at this age were secreting any androgens.

The most unusual feature of the prostate glands in all investigated opossums was the amount of glycogen found in all prostatic segments, especially in segment III. Mann (1964) reported that the reducing sugar in the semen of many mammals, including the opossum *Didelphis marsupialis*, is fructose. The cells of accessory sex glands convert blood glucose into cell glycogen which by a system of enzymes is converted into fructose of seminal plasma. Because in segment III glycogen was found not only in the epithelial cells but also in the lumina of the tubular glands (Figs. 9, 10), the conversion to fructose should also happen there. The high acid phosphotase reactivity in the lumina of segment III supports this suggestion (Fig. 6). Other enzymes necessary for such conversion may be present in the secretory products of the other two prostatic segments. If this speculation is correct, the amount of seminal sugar in opossums does not depend on the immediate level of glucose in the circulating blood.

Phospholipids, which may be utilized in sperm metabolism were found in segments I and III of all investigated opossums but their histochemical reactivity was strong only in *Didelphis marsupialis*.

The third segment in all species had higher catalase activity than that observed in the livers of these animals (Table 3). Prostatic catalase, if present in the ejaculate, will prevent injury to spermatozoa by hydrogen peroxide. Biochemically the prostate gland of the opossum is just as complex as that of eutherian mammals (Table 1); however, the physiological roles of prostatic secretions in opossums are not known. Howarth (1950), using electrostimulation, obtained about 30cc of semen from the Australian marsupial *Trichosurus vulpecula* (brush possum). After the prostate was cut off, the same stimulation produced only a small quantity of fluid from the Cowper's glands. Howarth concluded that the prostate gland supplied the fluid for spermatozoa during ejaculation.

The ejaculate, at least in some Didelphidae, coagulates and forms copulatory plugs (vaginal plugs) located in the lateral vaginal canals of the female — (Hartman 1924, Biggers 1966, Barnes 1977). Hartman observed that though these canals, during the female's receptive period, were greatly distended with fluid, they were completely filled by the copulatory plugs following coitus. The author

was so amazed by the mass of the two plugs, which in his opinion was greater than the combined size of all male accessory sex glands, that he concluded that the copulatory plugs in *Didelphis marsupialis* were formed by the fluid content of the lateral vaginal canals of the female, coagulated by the secretions from the seminal plasma of the male. Hruban *et al.* (1965) failed to obtain coagulation by mixing together secretions from different prostatic segments of *Didelphis marsupialis*. Barnes (1977) reported that freshly deposited copulatory plugs in *Marmosa robinsoni* females can be localized by palpation and was successful in getting coagulation by mixing the secretion from the larger pair of bulbourethral glands with luminal contents of prostatic segment I. Both, Hartman and Barnes noted that the spermatozoa-rich fluid part of the ejaculate was located above the plugs, and Hartman speculated that just as in rodents, plug formation prevented the outflow of spermatozoa from the female genital tract. Martan and Shepherd (1976) through experimentation with copulatory plugs demonstrated that in the guinea pig, the copulatory plug ensures the sole paternity of the first male to mate, presumably the dominant male, and suggested that this may be the role of the plug in other mammals.

There are 3 pairs of Cowper's glands (bulbourethral gl.) in *Didelphis marsupialis* (Fig. 1). The morphology of these glands in adult males was described by Chase (1939), and Rubin (1944). The latter author also studied the development of these glands in the pouch males. The greatest lobes (Cowper's 1,) were enclosed by a layer of striated muscle surrounding the connective tissue capsule from which septa extended between the glandular tubules. The middle lobes (Cowper's 2,) and the smallest lobes (Cowper's 3,) had a common striated muscle enclosure but their glandular portions were separated by individual connective tissue capsules. All three pairs of Cowper's glands were of the compound tubular variety and their ducts ran parallel to the lumen of the urethra before joining into common duct emptying into the cavernous urethra. The authors described the secretion as eosinophilic and homogenous in all three glands. The individual ducts were lined with epithelial cells similar to those lining the secretory tubules and were filled with secretion.

After castration all lobes declined in weight, their secretory tubules became smaller and the inter-tubular connective tissue between those tubules wider (Chase 1939). These changes became visible in the largest lobes at 20 days, in the intermediate lobes at 65 days, but in the smallest lobes there was no change, even at 90 days after the operation. Rubin (1944) observed primordia of Cowper's glands 5 days after birth as paired bilateral thickenings of the solid urethral plate. At 32 days these primordia had already separated into 3 individual pairs of prospective adult glands. Between 75-84 days the glands were surrounded by a muscle layer and their epithelial cords had acquired lumina, except in the smallest lobes. However, Cowper's 3 glands subsequently showed a rapid developmental spurt and at 125 days all lobes exhibited characteristics of compound tubular glands, including secretory activity.

Treatment of younger pouch males (5-39 days) with androgens caused a precocious gain in the size of Cowper's glands, and advanced differentiation of secretory tubules and canalization of the ducts. If treatment was prolonged, there was an inhibition of morphological development (Rubin 1944). In males older than 70 days, androgens stimulated normal development and resulted in glands comparable to those of much older untreated males. Gonadotrophins stimulated

Cowper's gland differentiation in young males of all ages while castration had no effect on normal development of the glands up to 100 days. Treatment with estrogens produced hyperplasia and abnormal development of Cowper's glands in young males. According to Rubin (1944), bulbovestibular glands (Bartholin gl.) of the opossum female are equivalent to the smallest pair of lobes (Cowper's 3,) in the males.

Simple techniques have been used to examine the histochemistry of the Cowper's glands secretions (Martan, unpublished observations). Secretory material in all lobes showed strong reactions for neutral and acid mucopolysaccharides and weak reactions for general protein. Secretions of the two smaller pairs of lobes were strongly positive for sulfated acid mucopolysaccharides. In the smallest lobes, the homogenous secretory material was mixed with unstained vacuoles. In the middle lobes, similar homogenous material contained globular bodies of various sizes. In the largest lobes, the secretion was homogenous and without either globules or vacuoles. In lumina with little secretion, the material in all lobes was granular rather than homogenous. Glycogen was not detected in any of the lobes by the techniques used.

In *Didelphis marsupialis* males the only accessory sex glands present were those originating from the embryonic urogenital sinus, namely the prostate gland and Cowper's glands. Even the urogenital sinuses of female embryos were competent to differentiate into these glands either under natural conditions (bulbovestibular gl., Rubin 1944) or under hormonal stimulation (prostate gl. Moore 1941). It is of interest that in didelphids and other marsupials (Tyndale-Biscoe 1973), vasa deferentia do not cross the ureters to open into the urethra (Fig. 1). Further, the accessory sex glands which in eutherian mammals originate from the embryonic Wolffian duct (seminal vesicles and ampullary gl.) are absent.

Rodger and Hughes (1973) in a detailed study of the accessory sex glands of the Australian marsupials separated species into two groups; those with a carrot-shaped prostate and those with a heart-shaped prostate. They reported that all prostates were of the diffuse type and segmented, and at least in some, these segments differed in the coloration. The authors named the segments anterior, central and posterior in the carrot shaped group, and dorsal and ventral in the heart shaped group. Histologically all segments consisted of simple tubular glands, lined with simple columnar epithelium. The height of epithelial cells was characteristic for segments and species. The secretion, at least in some segments, included round globules and spheres, some of which according to the authors originated from apocrine type of secretory activity of the epithelial cells. The histochemical analyses of all species investigated indicated that the chief secretory materials of the prostate glands were mucosubstances; though in some species, lipids were present. The authors noticed in several species similar segmentation of the prostate gland as reported for *D. marsupialis* (Chase 1939) and suggested that this type of prostate gland may resemble the ancestral marsupial pattern.

Barbour (1981) noticed apocrine secretion in his study of the prostate gland in the hairy-nosed wombat. Histochemical analysis of prostatic cells and their secretions revealed some proteins, and abundant iron and glycogen. The author was so impressed with the amount of glycogen secreted (especially in the posterior segment) that he considered glycogen to be the main product of this gland.

In the genital tract of the Australian marsupial males, Cowper's glands were always present and the number reported was from one to three pairs (Rodger and

Hughes 1973). The histology of Cowper's glands was more uniform among Australian species than that of the prostate glands, and similar to the microanatomy observed in *D. marsupialis* (Chase 1939, and Rubin, 1944). Histochemical studies revealed glycogen and lipids in the cells of the Cowper's glands of some species and the authors suggested that the glands generally secreted mucous material.

While the morphology of the prostate glands in the American and Australian marsupials appeared similar, biochemical studies of the seminal plasma, which is mainly the product of prostate gland (Howarth 1950), showed differences in composition of seminal sugars (Rodger and White 1973). In several species of Australian marsupials Rodger and White (1974, 1975a, 1976) reported N-acetylglucosamine and glucose to be the principal seminal sugars while fructose (the main sugar in the semen of eutherian mammals) was practically absent. In *Didelphis marsupialis*, Mann (1964) reported fructose, secreted by the prostate gland, to be the principal sugar in the seminal plasma. If fructose will be confirmed as the principal sugar in the seminal fluid of other members in the family Didelphidae, it will represent a significant difference in sperm metabolic requirements between Australian and American marsupials, and establish the first uniform difference between Australian marsupials and the eutherian mammals. According to Rodger and White (1975a), in Australian marsupials with the carrot shaped, three segmented type of prostate, the main site of glucose was the posterior segment, while a high level of N-acetylglucosamine was found in the central segment. Interestingly, the similar prostatic segments in *Didelphis marsupialis* (Martan and Allen 1965), showed a high glycogen content and multitudes of cytoplasmic granules. (Figs. 4, 7, 8, 9, and 10)

Biochemical studies of the prostate of the brush-tailed possum *Trichosurus vulpecula* (Cook et al. 1978) revealed high concentrations of zinc in the central segment, and a high activity of testosterone 5α reductase in the posterior segment of the gland. The authors suggested the possibility of testicular and epididymal androgen transport by blood vessels of the prostatic urethra and the prostate gland.

Little is known about the biochemistry of the secretory material produced by the Cowper's glands. The simple histochemical test done by Rodger and Hughes (1973) on the Australian marsupials and the present author on *D. marsupialis*, imply the existence of mucoproteins. Hearn (1975) reported great reduction in weights of prostate and Cowper's glands in 60 days castrated and hypophysectomized wallabies (*Macropus eugenii*), and Carrodus and Bolliger (1939) observed that injections of estrogen caused a decline in the size and weight of the prostate gland, especially in the posterior segment.

Setchell (1977) stated that in marsupials, coagulating glands were missing. However, copulatory plugs in the lateral vaginal canals in the females of some Australian marsupials (Kean 1959, Tyndale-Biscoe 1973) and some American marsupials (Hartman 1924, Biggers 1966, and Barnes 1977) were found. Copulatory plug formation occurs also in some eutherian mammals in which the presence of coagulating glands was not established (Price and Williams-Ashman 1961). The latter authors discussed at length the confusion which exists in the nomenclature of different prostatic lobes in the eutherian mammals and regarded coagulating glands as a part of the prostate gland, comparable to the anterior lobes. Earlier, Engle (1926) expressed a similar opinion and looked upon the coagulating glands in the rat and guinea pig as proximal lobes of the prostate glands and not as a distinct organ as reported by Walker (1910).

The sequential segments of the disseminate prostate gland in the marsupials are regarded by some authors (Rodger and Hughes 1973) as an adaptation for the production of different secretions in the absence of the other accessory sex glands (seminal vesicles and ampullary gl.). Other workers (Price and Williams-Ashman 1961) considered the difference between these segments as comparable to those observed in the prostatic lobes of eutherian mammals. Much more information must be gathered before any comparison in prostatic secretions between eutherian mammals and marsupials can be made. However, if one wishes to speculate, the recent report by Barnes (1977) that secretory products of the prostatic segment I in *Marmosa robinsoni* coagulated the secretion of the larger pair of Cowper's glands would point to this segment as functionally comparable to the anterior lobes of the prostate (coagulating gland) of the eutherian mammals. In addition to the above-mentioned copulatory plug formation, secretion of the prostate gland and Cowper's glands provides a vehicle to carry spermatozoa during ejaculation (Howarth 1950) and a favorable medium for their survival and function in the female genital tract (Price and Williams-Ashman 1961).

The ductuli efferentes in *Didelphis marsupialis* form a wedge-shaped body above the vascular plexus of the hilus of the testis (Fig. 11). In the adult males, this segment of the genital tract, is of yellowish-green color and is separated from the epididymis by a shallow groove (Martan *et al.*, 1967b). Chase (1939) reported that the testis was connected to the epididymis by a single efferent ductule while Ladman (1967) identified up to three ductuli efferentes in *D. marsupialis*. The simple columnar epithelium of these ducts, resting on the prominent basement membrane, was composed of ciliated cells and cells with short microvilli according to the latter author. This disagreement among experts may be explained by the observation that a single efferent duct divides into three to four ductuli in *Didelphis azarae* (Noqueira *et al.*, 1977).

Martan *et al.*, (1967b.) found that the highly convoluted ductuli efferentes, surrounded by abundant stroma, had wider lumina in their middle portion than at either end (Fig. 12) and reported low columnar and basal cells in their epithelia. Apical parts of the columnar cells showed a strong alkaline phosphatase reaction (Fig. 16), and contained granules of different sizes in some of which histochemical reactions denoted materials containing mucoprotein (Fig. 17) and possibly phospholipids. Ferric ferricyanide and dihydroxy dinaphthyl disulphide (DDD) methods indicated the presence of sulfhydryl groups in some of the granules (Figs. 14, 15). Some of the columnar cells had only microvilli, but others had both cilia and microvilli. The authors proposed that the epithelial cells of ductuli efferentes in *D. marsupialis* had absorptive and probably secretory functions; supported by Ladman (1967).

The wedge-shaped segment containing the ductuli efferentes from six months old opossum males did not have the yellowish green color of the adults (Martan *et al.*, 1967b). This lack of specific coloration and the absence of apical granules giving positive reactions with DDD and ferric ferricyanide in the epithelial cells, prompted the researchers to suggest that both, the coloration and the granules, were under testicular hormone control and associated with maturity.

The anatomical and histological structures of the ductuli efferentes of the four-eyed (*Philander opossum*, Brisson), murine (Marmosa sp., Gray), and woolly (*Caluromys derbianus*, Allen) opossums were similar to those described for adult *Didelphis marsupialis* (Martan *et al.*, 1967b.). However, their coloration was a

grayish pink and the apical granules, which give a positive reaction for sulfhydryl groups, were missing. In the columnar cells of the ductuli efferentes in the four-eyed opossum, another type of specific structure was observed in their apical cytoplasm (Figs. 19, 20). This single basophilic body, not limited by a membrane, was 1.5μm in diameter and contained RNA and protein. Thus the only observed differences in ductuli efferentes among the representatives of four genera of the family Didelphidae were on the cytological and cytochemical level (Figs. 13, 14, 17, 19). These differences probably represent the modified requirements of spermatozoa in different species. The lack of studies on the ductuli efferentes in the Australian marsupials prevents comparison between the two groups.

The epididymal duct in *Didelphis marsupialis*, with its anatomical divisions into caput, corpus, and cauda, resembled the epididymides of eutherian mammals (Snydle 1975). Using histological criteria, Snydle divided the epididymis of the opossum into eight segments. He observed that in the middle of the corpus (segment 5), single spermatozoa suddenly associated into pairs. In each pair, the two spermatozoa were attached to one another by the fusion of limited areas of cell membranes overlying their acrosomes. This pairing of spermatozoa was accompanied by strong histochemical reactions for alkaline phosphatase, polysaccharides, and acid mucopolysaccharides. The reactions occurred in both, the cytoplasm of the epididymal cells lining segment five and in the lumen of the duct. Electron microscopic studies have shown changes in the spermatozoan plasma membranes occurring in the same segment. Barnes (1977) reported that in *Marmosa robinsoni*, pairing of the spermatozoa occurred in the cauda epididymis. Pairing of spermatozoa is a common feature in American opossums and unknown in Australian marsupials. The functional aspects of the pairing of sperm are only speculative. However, in the guinea pigs, formation of another type of spermatozoan association, called rouleaux, was reported to be dependent on testicular androgens and not necessary for the sperm fertility (McGlinn *et al.*, 1979). On the other hand, spermatozoa in rouleaux were protected against phagocytosis by leukocytes in the female uterus while single spermatozoa were not protected (Martan and Shepherd 1973). High levels of serotonin were found in the epididymides and ductuli efferentes of *Didelphis marsupilais*, accompanied with even higher levels of an unknown, probably indole-like compound (Anderson *et al.*, 1979). Positive histochemical reactions were observed in the interductular connective tissue, mast cells, apical parts of epididymal epithelial cells, and subepithelial bands (Figs. 22, 23, 24, 25, 26). The cells forming these subepithelial bands had a high RNA content (Fig. 28). The authors proposed that the bands represented a myoepithelial layer under the epididymal epithelium (Fig. 27). If a myoepithelial layer will be observed to be characteristic of the epididymides of other marsupials, it will represent a distinct difference between the genital tract of marsupials and that of eutherian mammals. Chase (1939) described the ciliated columnar epithelium in the epididymis and the vas deferens of *Didelphis marsupialis*. The cilia in the epididymal duct were not confirmed by any other researcher and there is no detailed report on the vas deferens of any marsupial.

The epididymides of the Australian marsupials had anatomical divisions similar to those of eutherian mammals (Cummins 1980), and the passage of spermatozoa through the epididymal duct was estimated to last between eleven and thirteen days (Setchel and Carrick 1973). In *Trichosurus vulpecula* the epididymis had higher testosterone 5 α-reductase activity than the testis (Cook *et al.*, 1978),

and spermatozoa acquired their progressive motility in the distal corpus epididymis (Temple-Smith and Bedford 1976).

The elimination of unused spermatozoa by spermatorrhea, first reported in Australian marsupials (Carrodus and Bolliger 1939, Bolliger 1942, Woolley 1964) and later found in some American species, (Biggers 1966, Barness 1977) appears to be another similarity between the two marsupial groups and in contrast to the majority of eutherian mammals. The occurrence of both spermatorrhea and coagulation of the ejaculate in marsupials led Rodger and White (1975b) to speculate that the mechanism of ejaculation in this group is different from that of eutherian mammals.

In conclusion, the marsupial male genital tract is structurally more stereotyped than that of his eutherian counterpart. The accessory sex glands originating from the embryonic urogenital sinus are present, while accessory sex glands originating from the embryonic Wolffian ducts are missing. All prostate glands so far reported were of the diffuse (disseminate) type. Comparative studies when done, have confirmed the morphological uniformity among the members of the groups studied. Differences when reported were on the subcellular and cytochemical levels only. The products of marsupial accessory sex glands coagulate when ejaculated, forming vaginal plugs. Plugs have been observed in both the American and Australian marsupials. Similarly, there have been several reports of spermatorrhea in both groups, which suggest functional similarities of marsupial sex glands and ducts.

Additional information on the genital ducts of marsupials is needed before any comparison within this group and with eutherian mammals can be made. Pairing of spermatozoa in the epididymal duct of American marsupials provides a distinct example of differences between them and the Australian group. Reports that the principal sugars of seminal plasma in Australian marsupials were glucose and N-acetylglucosamine rather than fructose, established a significant difference between this group and the eutherian mammals. On the other hand, if fructose will be confirmed as the main sugar in the seminal fluid of American marsupials, an additional difference between the two marsupial groups will become evident. However, apocrine type of secretion and an unusually high amount of glycogen reported in prostatic secretions in both the American and Australian marsupials point to their functional similarity.

The morphological and physiological patterns of the marsupial male genital tract seem to be emerging. Many more species need to be studied before more definite conclusions are drawn.

ACKNOWLEDGEMENT

The author gratefully acknowledges Dr. Benjamin A. Shepherd for his assistance in reading and proofing of the manuscript.

Table 2.

Oppossums	Body weight in grams	Prostate weight in grams	Prostate weight as % of body weights*	Colors and weights of the individual segments expressed as % of total prostate weight*					
				Segment I		Segment II		Segment III	
North American***	1160-5280	7.4-13.0	0.26 ± 0.03 (13)	11.3 ± 1 (8)	light brown	53.6 ± 1.8 (8)	pink	35.1 ± 2.1 (8)	dark grey
Four-eyed	345-1100	0.67-2.0	0.19 ± 0.01 (7)	9.8 ± 1.1 (3)	pale grey	57.0 ± 1.3 (3)	pink	33.2 ± 0.5 (3)	dark grey
Woolly	333-362	1.67-2.19	0.56 ± 0.02 (4)	40.2 ± 5.4 (4)	pale grey**	47.9 ± 5.6 (4)	pink	11.9 ± 1.8 (4)	dark grey
Murine	45-80	1.1-2.15	2.71 ± 0.44 (6)	17.1 ± 1.3 (5)	pale grey	26.5 ± 1.6 (5)	pink	56.5 ± 1.5 (5)	dark grey

*Numbers in brackets denote number of animals studied.
The ± figure is the estimated standard deviation of the mean.
**Segment I of woolly opossum had gelatinous consistency.
***After Hruban *et al.*, 1965 and (unpublished data.
From J. Martan *et al.*, 1967a., unpublished manuscript)

Table 3. Catalase and Uricase Activity of Livers and Prostates

Opossums	Catalase*				Uricase*			
	Liver	Segment			Liver	Segment		
		I	II	III		I	II	III
North American**	2.58 ± 0.43 (9)	1.37 ± 0.47 (5)	0.41 ± 0.08 (3)	35.36 ± 4.28 (6)	0.91 ± 0.06 (9)	0-trace (6)	0-trace (3)	0-trace (6)
Four-eyed	4.38 ± 0.13 (4)	10.23 ± 5.72 (3)	0.67 ± 0.11 (3)	166.2 ± 22.5 (4)	0.55 ± 0.01 (4)	0.42 ± 0.09 (3)	0.49 ± 0.12 (3)	0-trace (4)
Woolly	1.48 ± 0.41 (4)	4.07 ± 3.04 (3)	0.32 ± 0.08 (2)	16.62 ± 2.32 (2)	0.20 ± 0.03 (2)	trace (3)	0-trace (3)	0-trace (3)
Murine	5.10 ± 1.07 (4)	—	1.66 (1)	26.23 ± 7.88 (3)	0.95 ± 0.18 (4)	—	0 (1)	0-trace (3)

*Catalase and uricase activities are expressed in units per gram of nitrogen.
Numbers in brackets denote number of specimens from different animals.
The ± figure is the estimated standard deviation of the mean.

**After Hruban *et al.*, 1965 and (unpublished data.
From J. Martan *et al.*, 1967a., unpublished manuscript)

EXPLANATION OF FIGURES

Fig. 1. Male opossum, *Didelphis marsupialis virginiana* genital tract. (Redrawn and modified from C.R. Moore, Physiol. Zool. 14:1-45, 1941.)

Fig. 2 to 4. Longitudinal sections through the opossum prostate showing separation of the segments (I on the left, III on the right). The first segment shows a pale inner zone. The subdivision of the second and third segment into outer zone, middle zone and inner (secretory ducts) zone is evident in some stains. A thin superficial muscular capsule is recognizable in some areas. x 3.

2. Mallory hematoxylin stain.

3. Mitochondrial stain after Kull.

4. Periodic acid-Schiff reaction after diastase digestion and Alcian blue reaction.

Fig. 5. Transition between the outer (top and middle (bottom)) zone of the second segment. The pale cells in the outer zone probably represent C-cells. The dark cells in the middle zone are A-cells. Both zones contain moderately dense B-cells. Periodic acid-Schiff reaction. x 300.

Fig. 6. Acid phosphatase reaction on the third segment shows localization of the enzyme in villi and in lumina. x 300.

(Figs. 2, 3, 4, 5 and 6 from Hruban *et al.*, J. Exper. Zool. 160:81-105, 1965 Alan R. Liss, Inc.)

Fig. 7. A and B cells in the epithelium of segment II of the prostate gland. Type A cells are characterized by the presence of rounded secretory granules and a greater height than the type B cells which are typified by the presence of elongate secretory granules. Ludford osmium tetroxide method, paraffin embedded, 3 μm. x 1,750.

Fig. 8. Differential staining of A and B cells following fixation in 4.0% formaldehyde containing 1.0% cadmium chloride and refixation (after sectioning and mounting) for three hours in 5% mercuric chloride at 50°C. The rounded granules in the "A" cells stain red after treatment with the periodic acid-Schiff procedure while the elongate granules of the B cells stain purple after treatment with Mallory's phosphotungstic acid hematoxylin. Paraffin embedded, 2 μm. x 1,750.

Fig. 9. Distribution of glycogen in segment III of the prostate gland. Dense accumulation of this material are seen in the epithelium and large dense mass is present in the lumen. Periodic acid-Schiff reaction hematoxylin counterstain. Bouin fixation, 8 μm frozen section. x 700.

Fig. 10. Identical to figure 9 but with omission of hematoxylin counterstain. Large numbers of periodic acid-Schiff reactive globules are present in the glandular lumen. x 700.

(Figs 7, 8, 9 and 10 from Martan and Allen, J. Exper. Zool. 159:209-229, 1965 Alan R. Liss, Inc.)

Fig. 11. Gross appearance of the testis and epididymis of adult North American opossum. Cauda (C) of the epididymis is detached from the testis. Ductuli efferentes (arrow), epididymis (E), testis (T), vascular hilum (V). x 3.5.

Fig. 12. Section through the ductuli efferentes and adjacent epididymis (E). The ductuli efferentes near testis (arrow) have narrow lumina. Wide lumina are seen in the major portion of the ductuli efferentes (D). North American opossum, hematoxylin-eosin, 6 μm section x 30.

Fig. 13. Cross section of a ductulus efferens of the woolly opossum, showing distribution of large, periodic acid-Schiff reactive, diastase resistant, granules in epithelial cells. Lumen (L). Periodic acid-Schiff-Alcian blue reaction, 6 μm section x 2,000. (Compare with Fig. 17)

Fig. 14. Cross section of a ductulus efferens of the North American opossum. Ferric ferricyanide reaction demonstrating localization of granules with sulfhydryl groups. Lumen (L). 5 μm section x 530.

(Figs 11, 12, 13 and 14 from Martan *et al.*, 1967b. J. Morph. 121:81-101. Alan R. Liss, Inc.)

Fig. 15. Same as in Fig. 14. Sulfhydryl groups in granules demonstrated by DDD method. Connective tissue (CT). x 1,300.

Fig. 16. Distribution of alkaline phosphatase in cross-section of ductulus efferens of North American opossum. The reaction product is restricted to the apical region of epithelial cells. Lumen (L) 15 μm freezing microtome section. x 600.

Fig. 17. Distribution of periodic acid-Schiff positive, diastase resistant granules in the epithelial cells of ductuli efferentes of North American opossum. Nuclei (N). Periodic acid-Schiff-Alcian blue reaction, 6 μm section x 2,000. (Compare with Fig. 13)

Fig. 18. Localization of iron in the apices of cells and in cytoplasmic globules in lumina in segment I of the prostatic complex. Turnbull blue reaction x 300.

Fig. 19. Ribose nucleic acid revealed in basophilic bodies (arrows) stained with azure B reaction at pH 4.0. One body in lumen (L). Nuclei (N). 7 μm section x 2,000.

Fig. 20. Basophilic bodies (arrows) are positive with Luxol Fast Blue MBS, suggesting the presence of phospholipid material. Nuclei (N) 7 μm section x 1,500.

Fig. 21. Distribution of acid phosphatase in segment I of the prostatic complex. The reaction is restricted to apical portion of intact epithelial cells and cytoplasmic vesicles released into tubular lumina. Frozen section, Gomori method, x 565.

(Figs. 15, 16, 17, 19 and 20 from Martan *et al.*, 1967. J. Morph., 121:81-101. Alan R. Liss, Inc.)

(Fig. 18 from Hruban *et al.*, 1965, J. Exp. Zool. 160:81-105. Alan R. Liss, Inc.)

Fig. 22. Section through the cauda epididymis. Note the subepithelial band (arrow), epithelial granules (g) and interductular connective tissue x 528.

Fig. 23. Excitatory light quenching of induced yellow fluorescence. Compare with Fig. 22., x 528.

(Figs. 22 and 23, from Anderson *et al.*, 1979, J. Anat. 129:141-149 Cambridge U. Press)

Fig. 24. Section through the cauda epididymis of the opossum. Note the blood vessel (arrowhead) and mast cells arrow; x 300.

Fig. 25. Section treated with $NaBH_4$. Compare with Fig. 24. x 300.

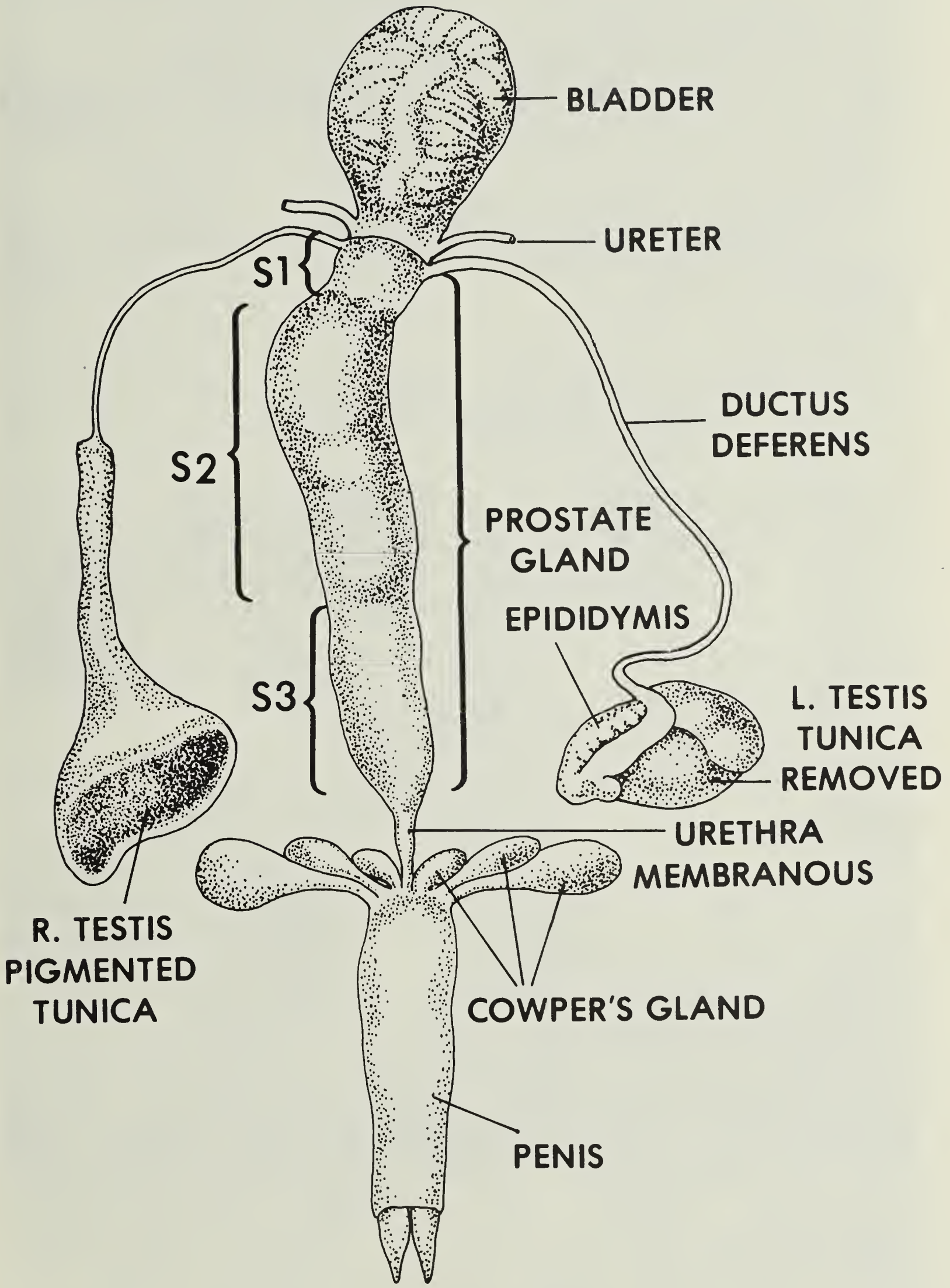
Fig. 26. Cuada epididymis following reinduction of fluorescence with gaseous formaldehyde. Compare with Figs. 24 and 25. x 300.

(Figs. 24, 25 and 26, from Anderson *et al.*, 1979, J. Anat. 129:141-149 Cambridge U. Press)

Fig. 27. Nuclei (arrow) in the subepithelial band of the opossum cauda epididymis, Feulgen, x 320.

Fig. 28. Cross-section of cauda epididymis of the opossum exhibiting RNA positive subepithelial band, methyl green-pyronin Y, x 400.

(Figs. 27 and 28, from Anderson *et al.*, 1979, J. Anat. 129:141-149 Cambridge U. Press)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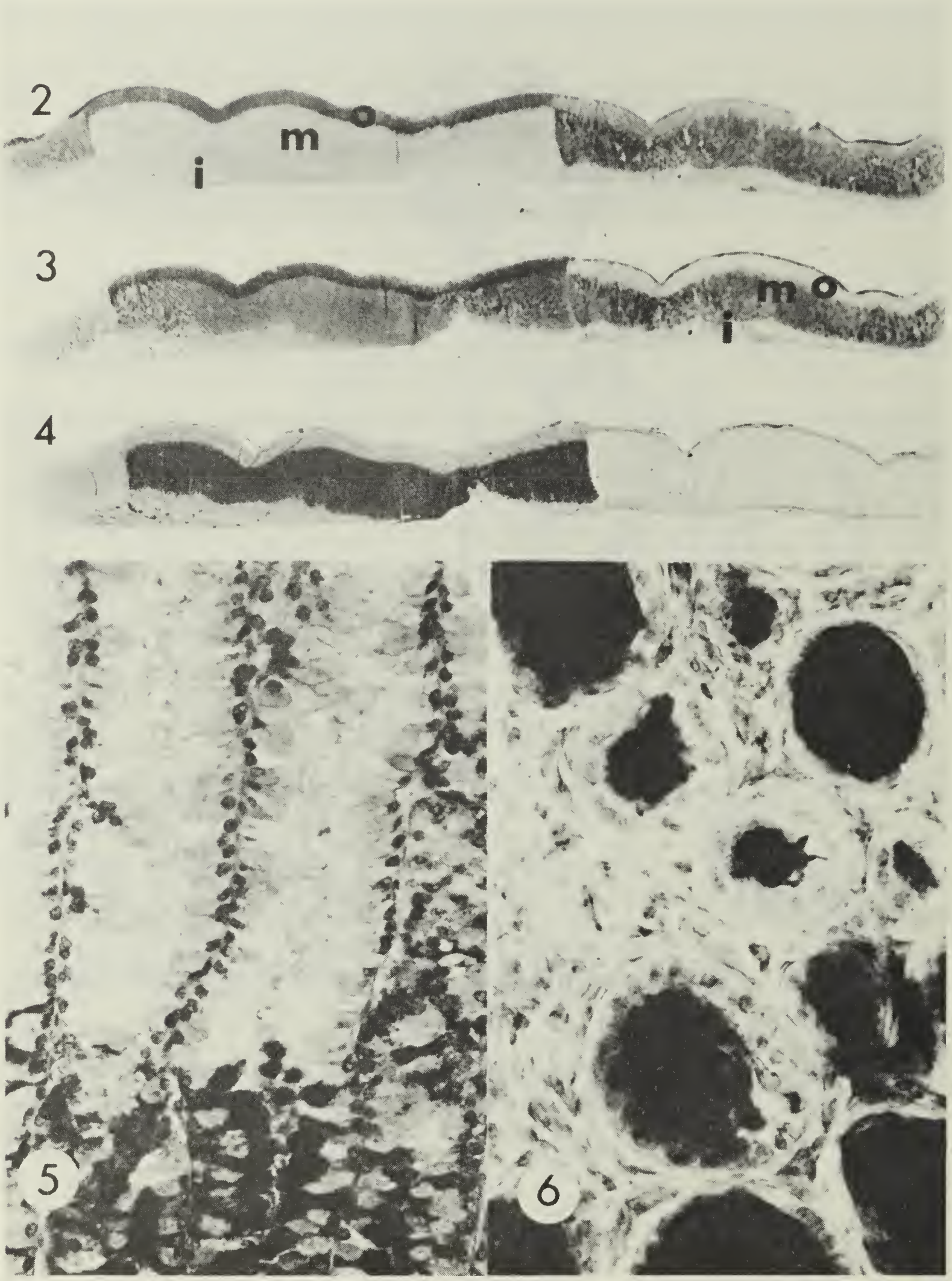
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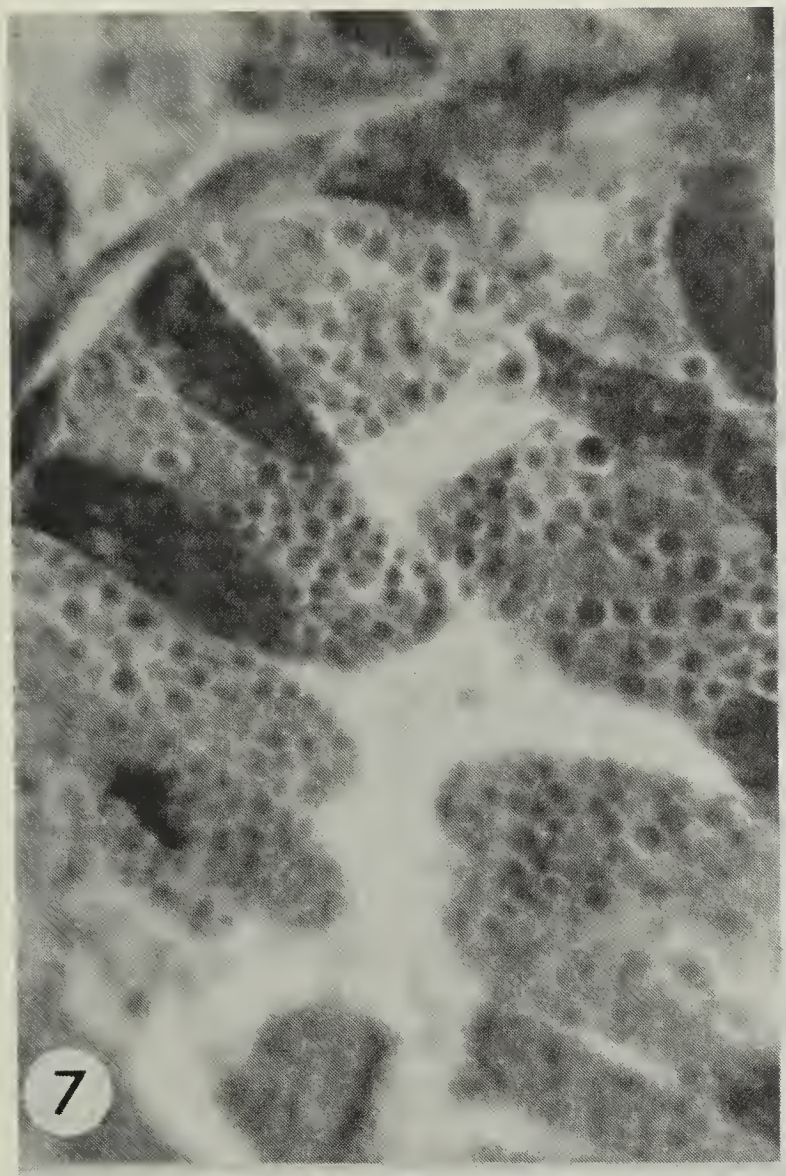
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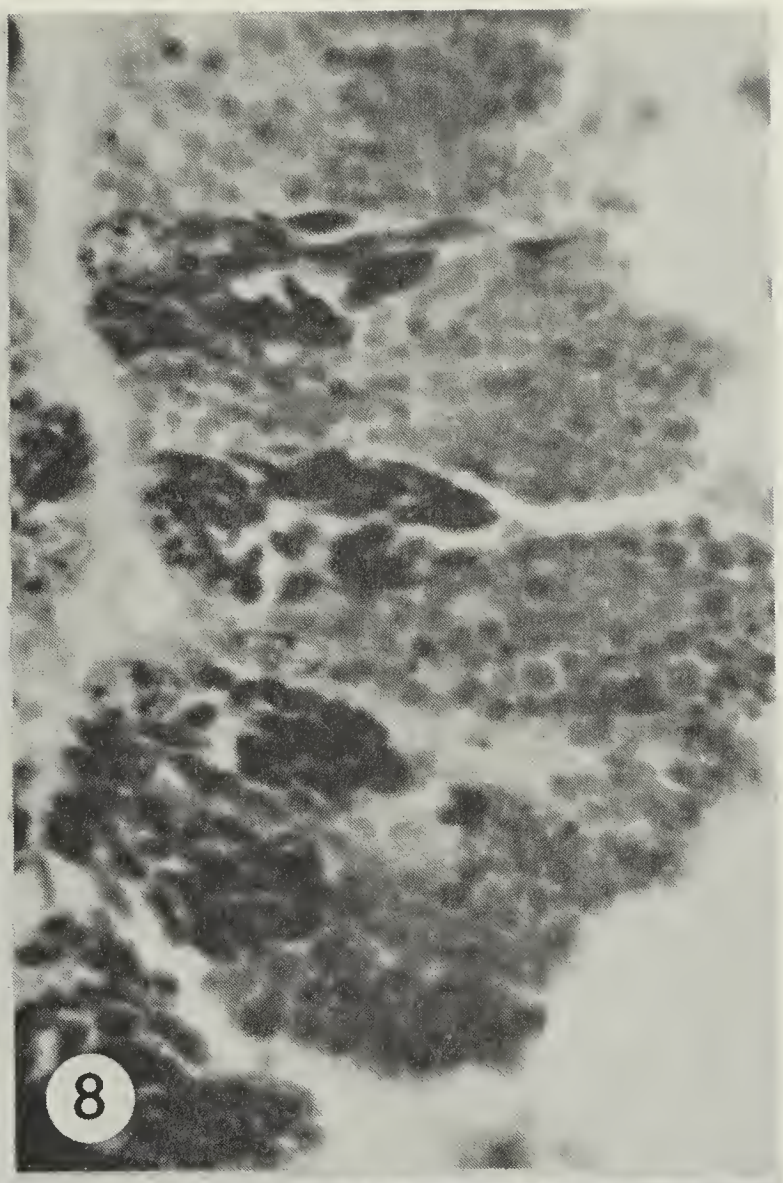
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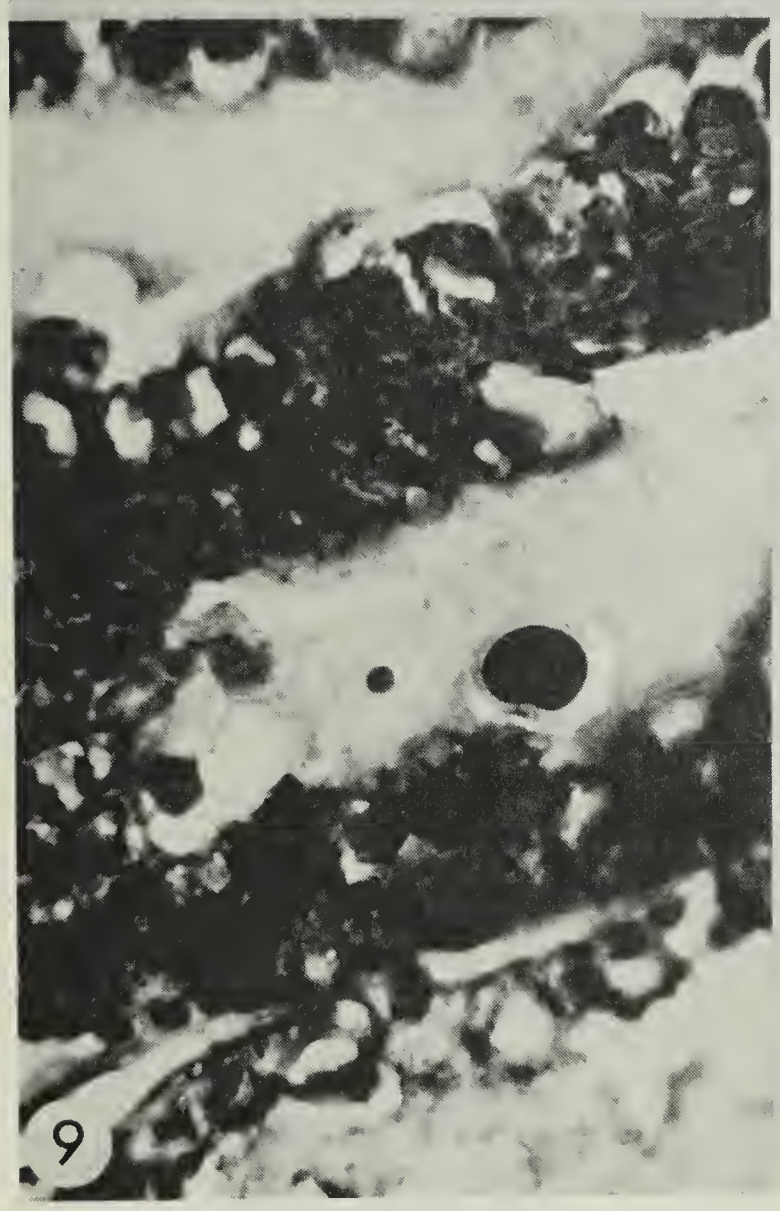
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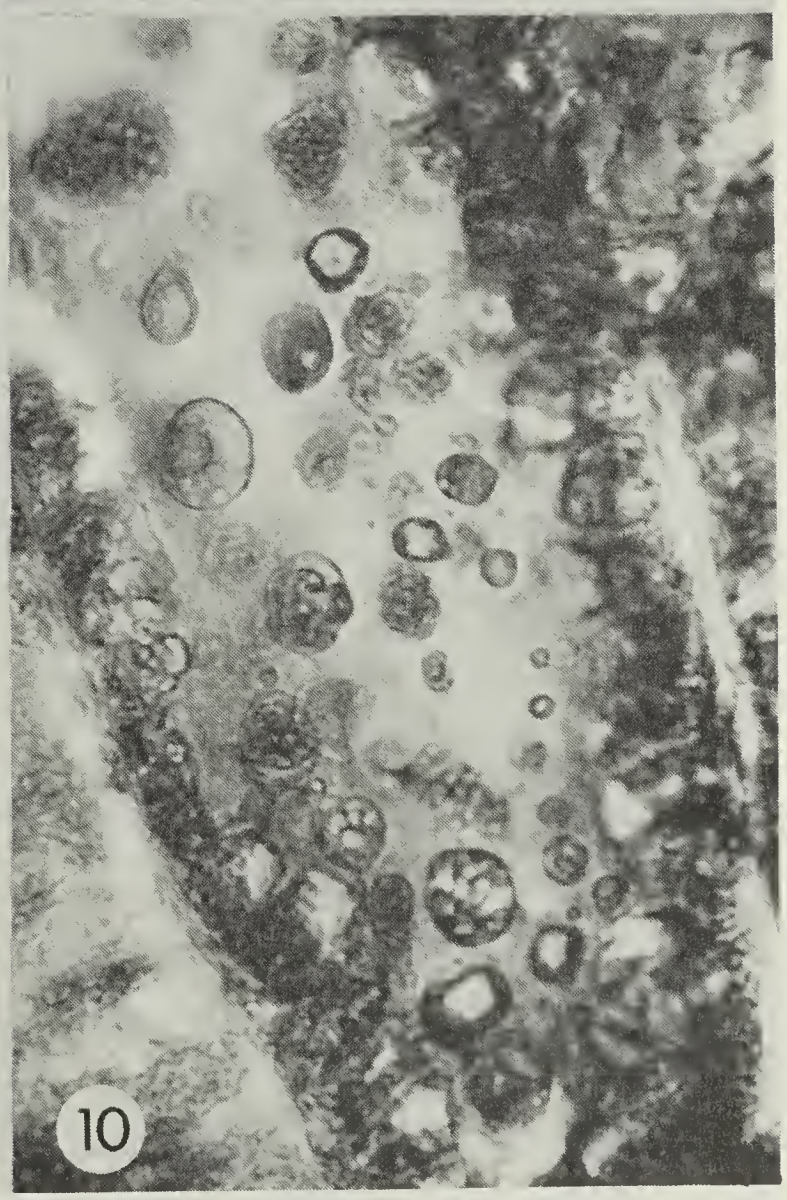
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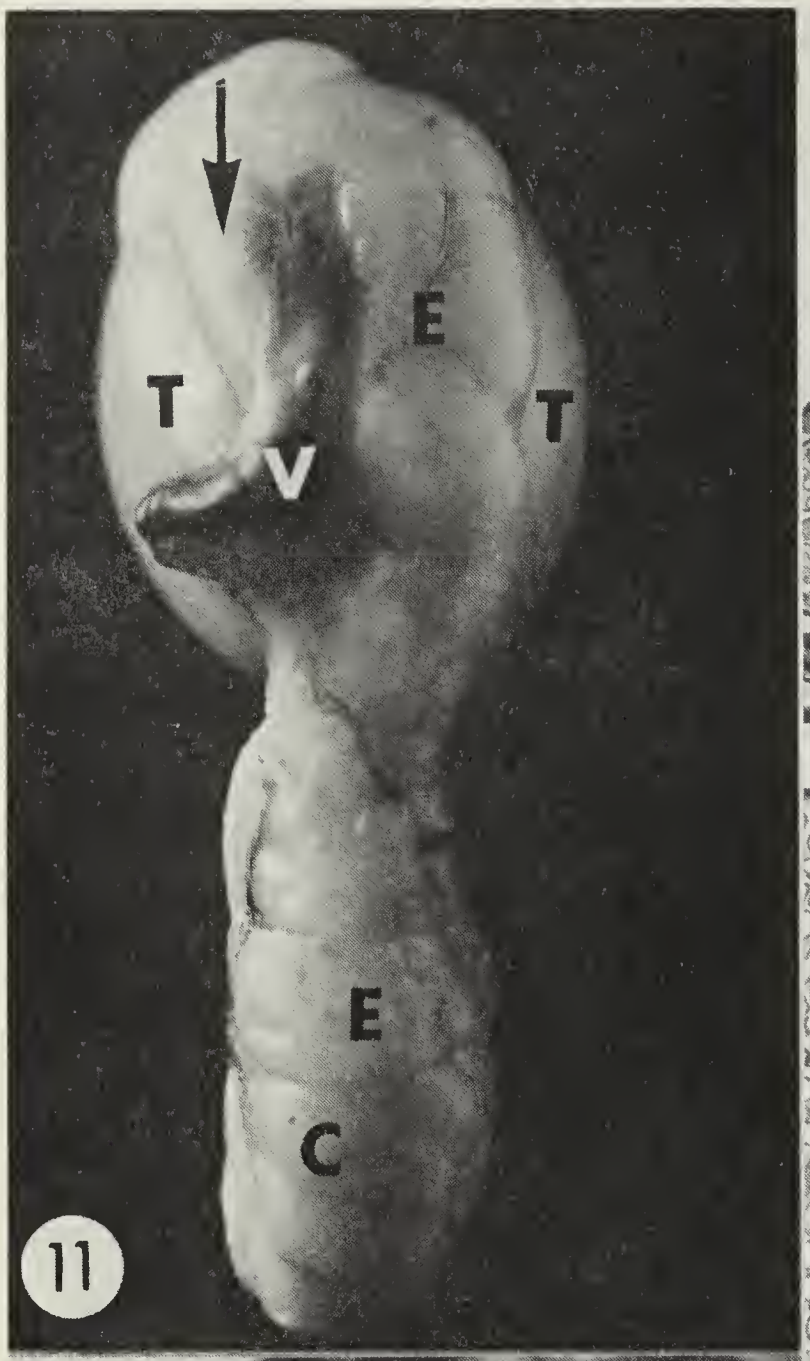

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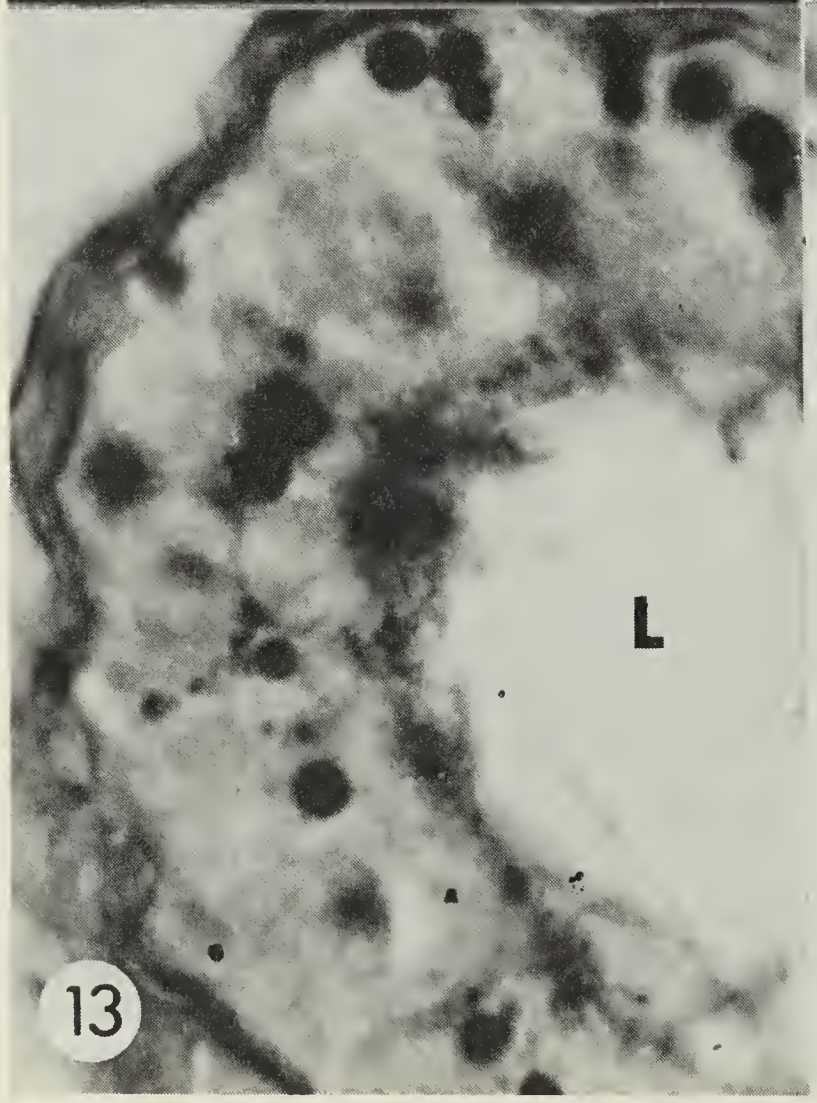

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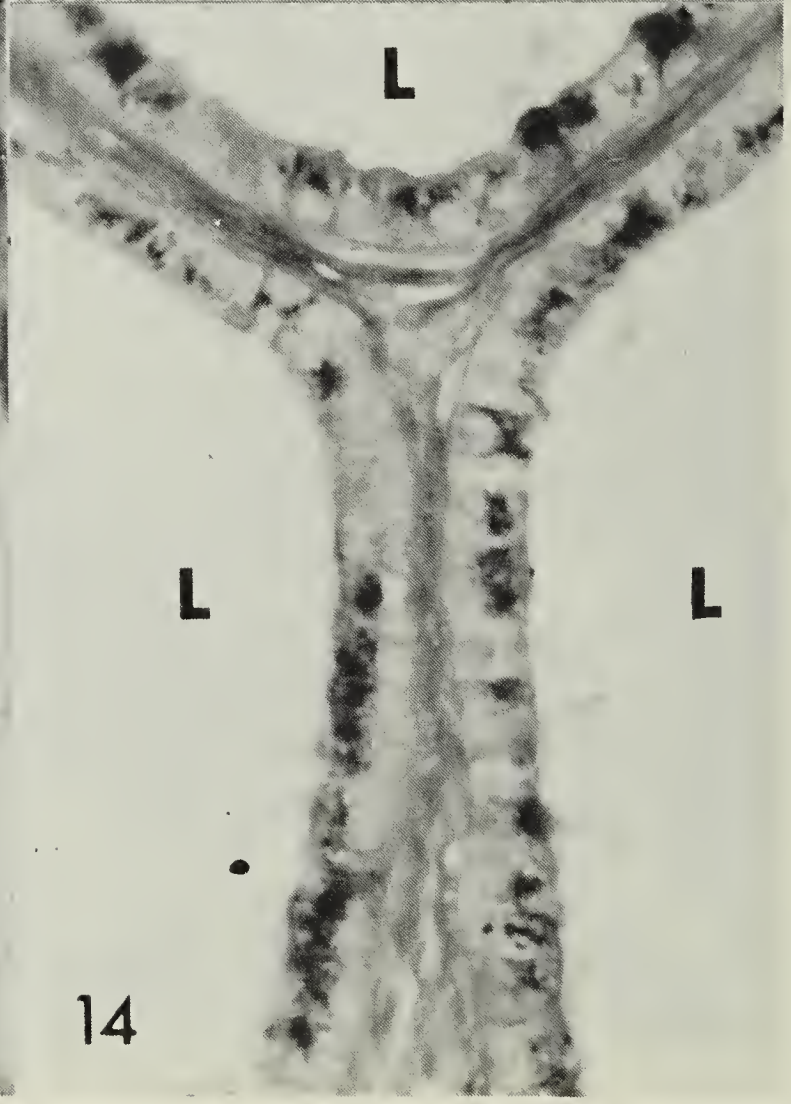

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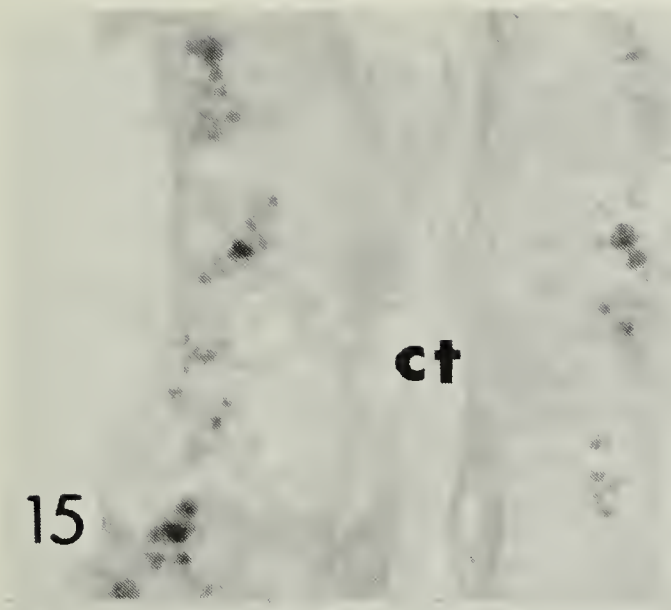

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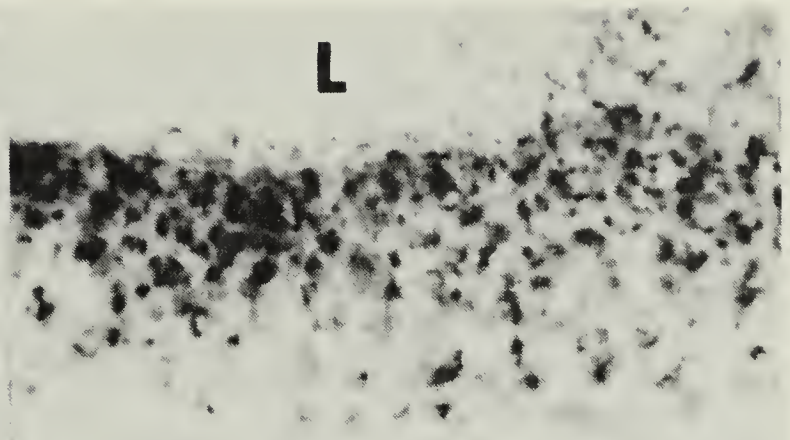

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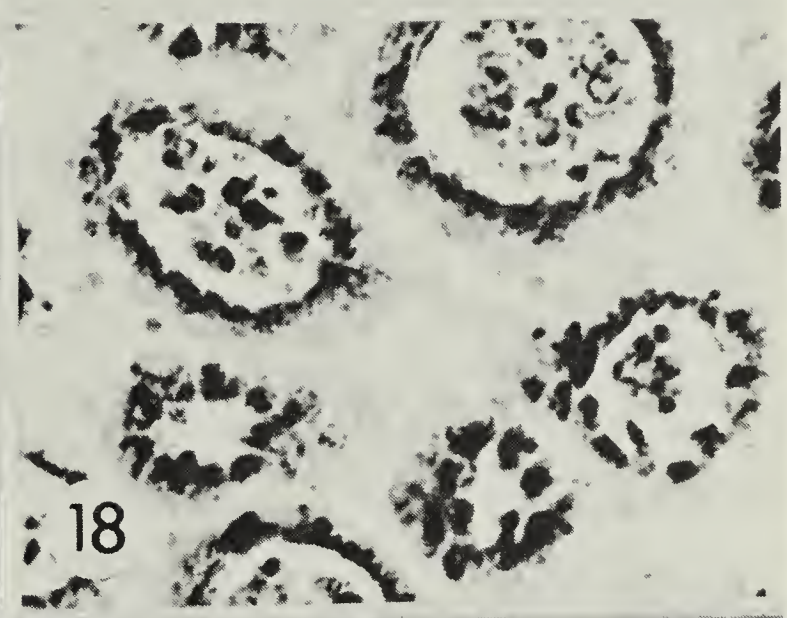

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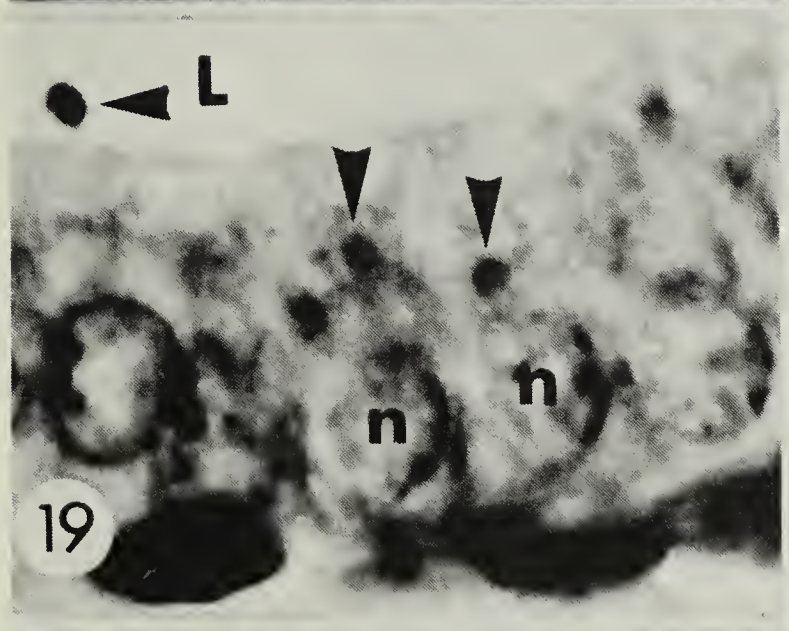

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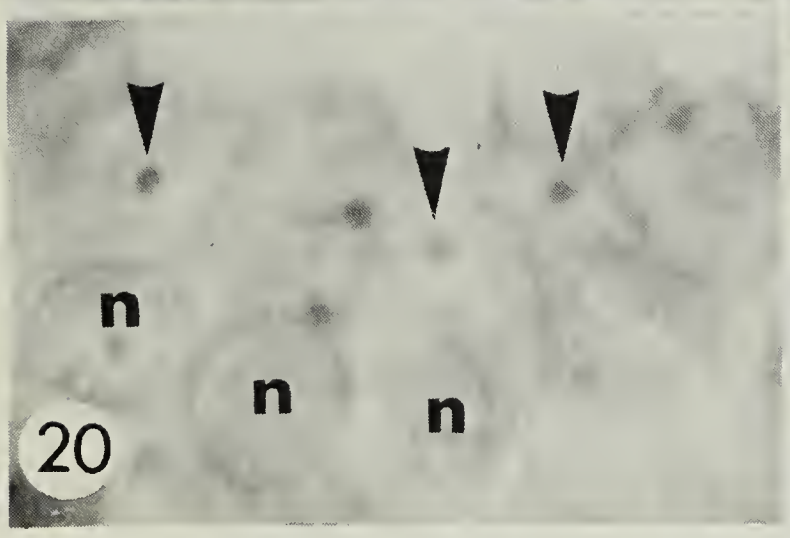

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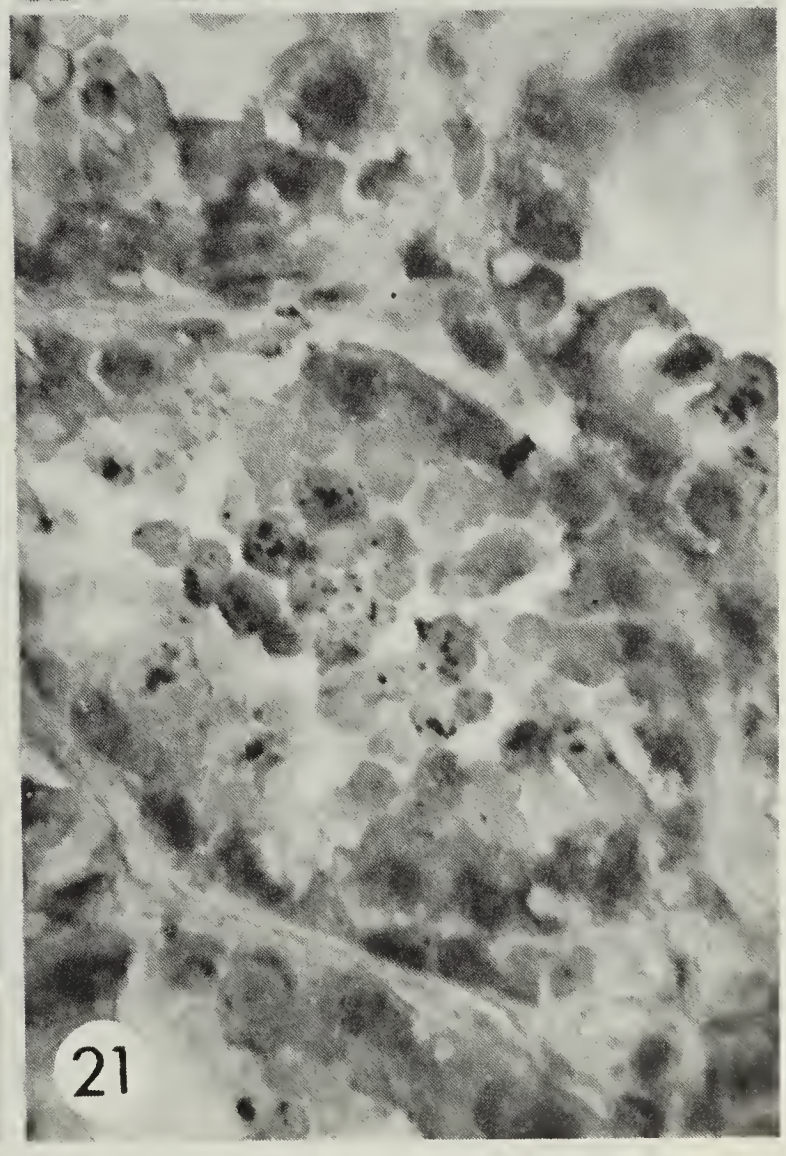

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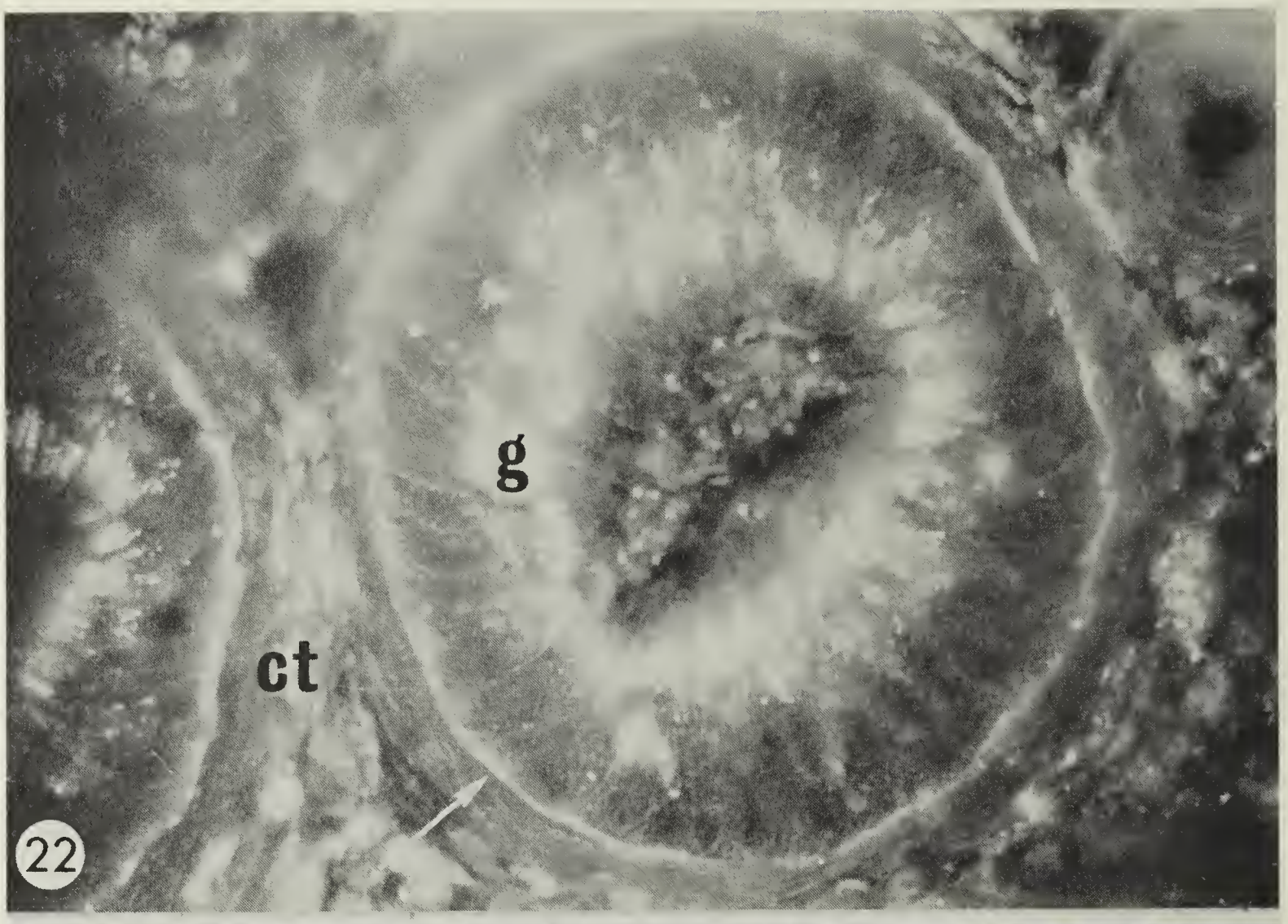
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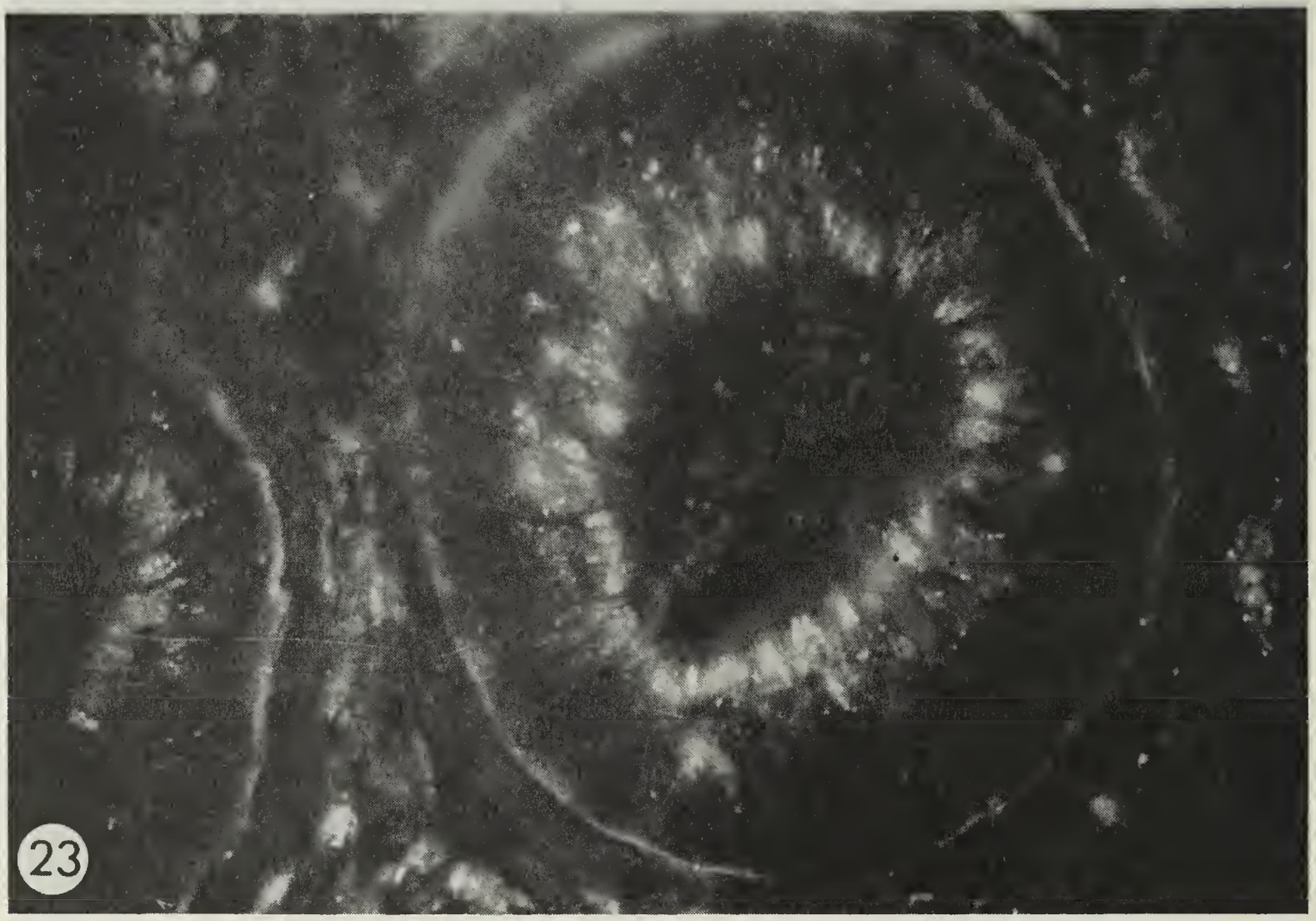
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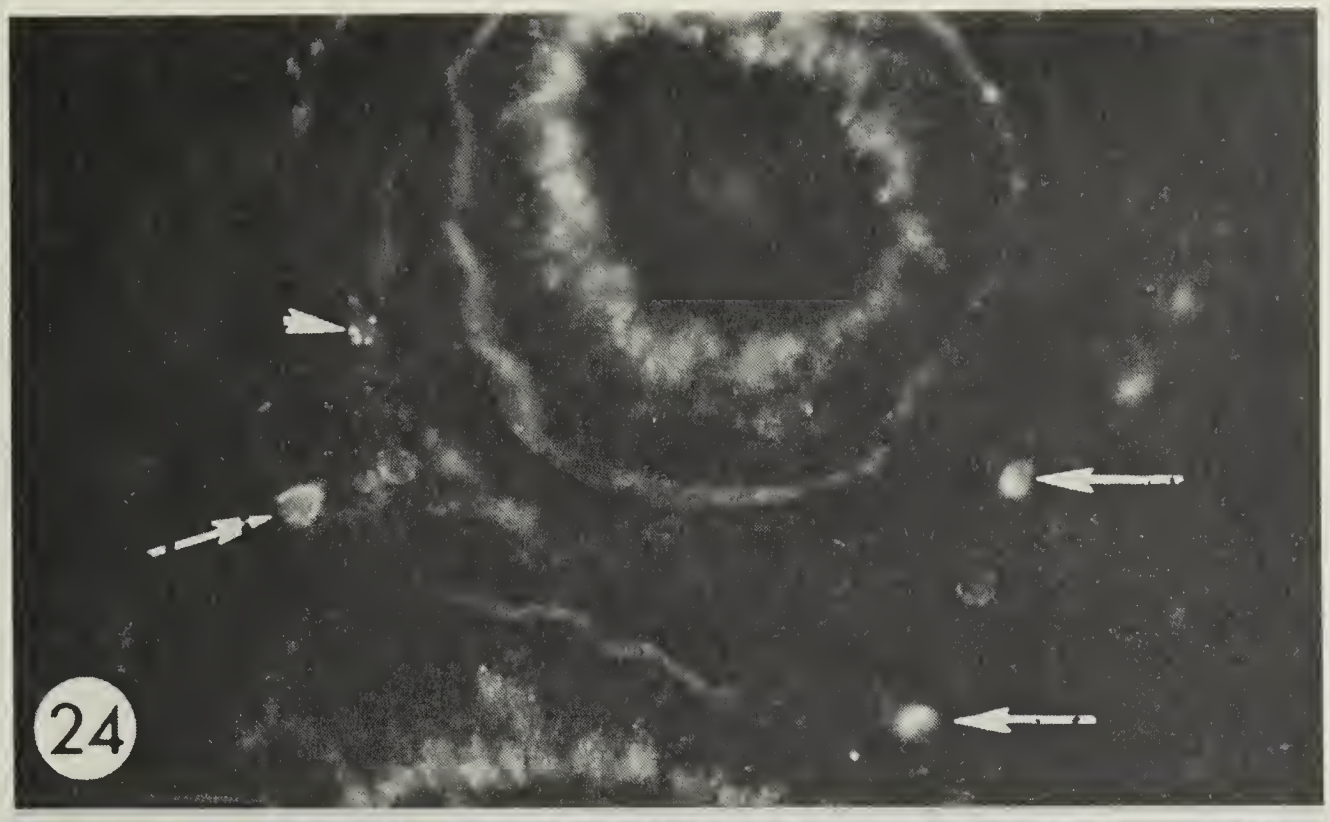

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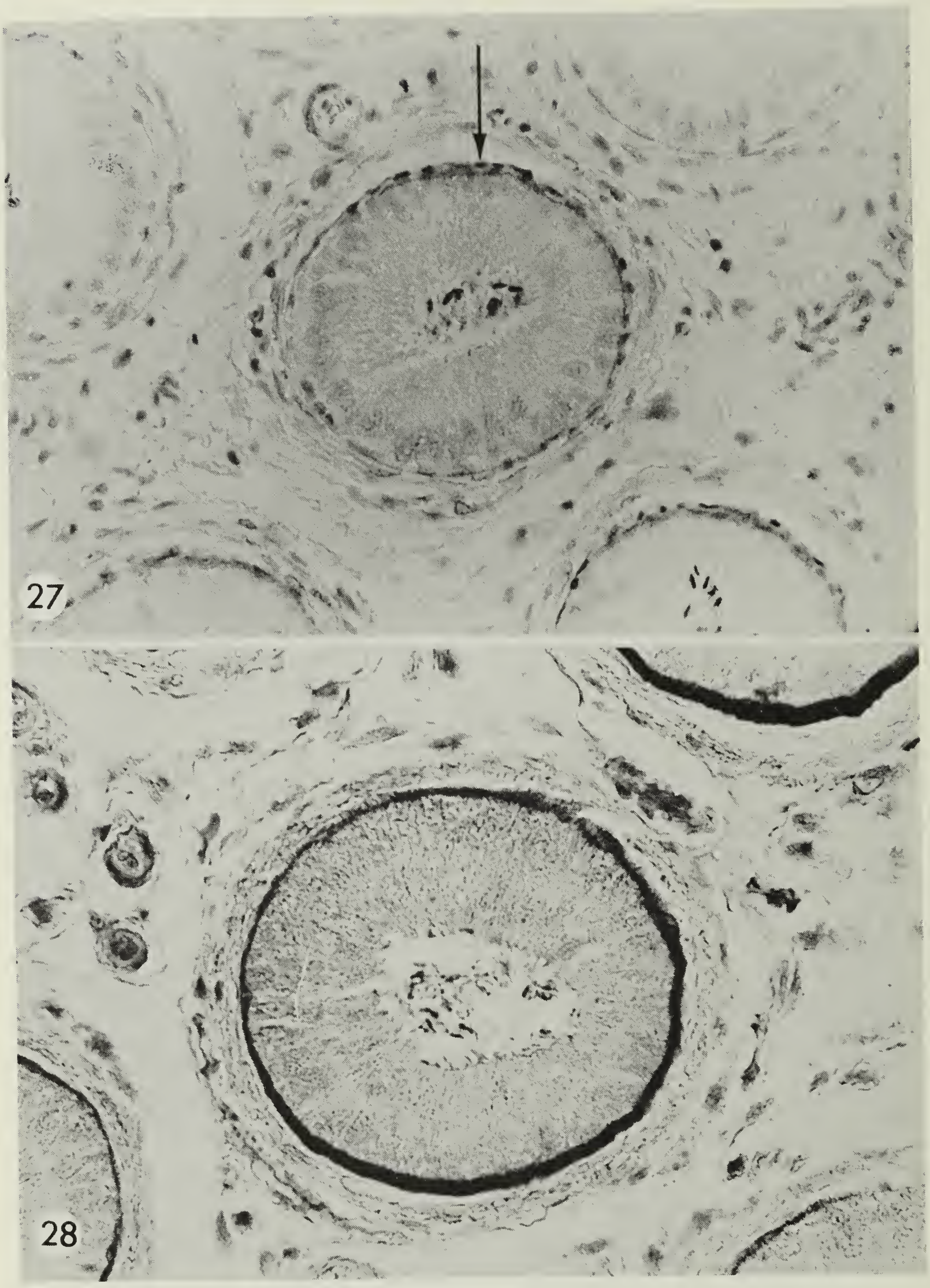
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Nexuses in Intestinal Smooth Muscle: The Use of a Simple Tracer Technique

Thomas S. Leeson
Department of Anatomy
University of Alberta
Edmonton, Alberta, Canada, T6G 2H7
and
C. Roland Leeson
Department of Anatomical Sciences
University of Illinois
Urbana, Illinois, U.S.A. 61801

ABSTRACT

Nexuses or gap junctions occur frequently between smooth muscle cells of the inner circular layer of the muscularis of the rat duodenum but apparently are not present in the outer longitudinal layer. While nexuses are visible and some details of structure can be seen in tissue routinely fixed and stained for electron microscopy, after lanthanum chloride treatment nexuses are electron dense and clearly visible at low magnifications. At higher magnifications, nexuses sectioned in all planes are identifiable whereas, in the main, nexuses in control material only are recognizable when cut perpendicular to the plane of the contact area, i.e. in nexuses showing a pentalaminar structure. In oblique and tangential ("en face") sections, connexons or particules are outlined by the lanthanum tracer and density and arrangement of packing of these particles is defined. The significance of these findings is discussed in relation to current knowledge of the nexus in smooth muscle and the gap junction in other cell types, and it is concluded that simple tracer techniques such as that utilized in this study still should prove useful in further studies of this type of cellular junction.

INTRODUCTION

One type of intercellular junction, the gap junction (or nexus), is concerned mainly with cell-to-cell communication and is believed to include intercellular

channels that mediate electronic coupling and the transport between cells of ions and small molecules (Bennett and Goodenough, 1978). Gap junctions are widely distributed in smooth muscle and other tissues and have been studied extensively by routine electron microscopy. Freeze fracture studies also have proved useful, particularly in respect of the surface distribution of the junctions, their shape and extent. In routine electron microscopy, gap junctions appear as areas of close apposition of the plasmalemmae of two adjacent cells with a slender gap of 2 to 3 nm between the membranes. While some intercellular tracers, such as lanthanum, penetrate this slender intercellular gap, the space is impermeable to other tracers such as horseradish peroxidase (Hopkins, 1978). When penetrated by such tracers the space also shows discrete bridges passing across the gap from one membrane to the other (Gabella, 1979). In sections where the plane of section is tangential to the cell surface and passes through a gap junction, 7 to 8 nm particles can be seen associated with the plasmalemmae, the particles often in hexagonal array. High resolution and X-ray diffraction studies have suggested that each particle or connexon unit is composed of six subunits forming a short cylinder with a central hydrophilic channel, these components of the opposed plasmalemmae being in register so that their hydrophilic channels are in continuity. Recently, a study of detergent-isolated gap junctions of mouse liver (Baker et. al., 1983), using negative staining with uranyl acetate, minimal irradiation methods and optical diffraction, has suggested that there are asymmetries in the gap junction with skewing of the six-lobed connexons. While all connexons may be identical chemically, their packing in hexagonal arrays on the two sides of the junction appeared to be nonequivalent.

In smooth muscle, gap junctions usually are called nexuses. Studies of intestinal smooth muscle to a great extent have been concerned with electrical coupling. An extensive study of electrically-coupled smooth muscles (Daniel et. al., 1976) showed that nexuses were common in some types but rare or absent in others. In the longitudinal muscle of dog intestine examined by thin section and freeze-fracture, nexuses were absent and it was suggested that in this tissue nexuses cannot account for electrical coupling. The freeze fracture appearance of the nexus in smooth muscle of several sites in different animals has been compared (Fry et. al., 1977). Usually, nexuses are oval, about 0.15 um^2 in surface area and, in the guinea pig sphincter pupillae muscle, numbered in the tens rather than the thousands per cell. The authors further discussed the minimum size of a nexus and concluded that, by freeze fracture, some with small aggregations of particles would not be recognized as nexuses in thin section. Further, they described the nexuses as being arranged in rows parallel to the main axis of the cell, usually alternating with longitudinal rows of plasmalemmal vesicles, and, in several locations, with a close relationship with sarcoplasmic reticulum. An extensive report (Gabella, 1979) has discussed cell junctions in the smooth muscle cell in relation to structural aspects of contraction. The author commented that nexuses in smooth muscle conform to the general structure reported for other tissues and confirmed previous reports that the number of nexuses varied considerably in different muscle. The sphincter papillae of the guinea pig shows numerous nexuses (Gabella, 1974; Fry et. al., 1977), as does the circular musculature of its ileum, where nexuses occupy about 0.2% of the cell surface (Gabella and Blundell, 1979). In the longitudinal muscle coat of taenia coli, nexuses are reported as small and few (Fry et. al., 1977), or extremely rare (Gabella, 1976). While in the cat small intestine nexuses are present in both layers of muscle (Taylor et. al., 1977), they are absent in longitudinal muscle of

dog duodenum (Henderson et. al., 1971), dog stomach (Daniel et. al., 1976) and guinea pig ileum (Gabella and Blundell, 1979). Gabella (1979), also suggested that in some tissues electrical coupling is achieved by a mechanism not involving gap junctions.

Gabella and Blundell (1979) studied the circular musculature of guinea pig ileum by freeze fracture to analyze quantitatively the nexuses between its smooth muscle cells. They reported an average cell surface area of 5,074 um^2 with a packing density of nexuses of 48 per 1000 um^2 of cell surface or about 244 nexuses per cell, a figure higher than an earlier estimate by Fry et alia (1977) for the sphincter pupillae muscle. Gabella and Blundell (1979) also described nexuses as varying in area from less than 0.1 to about 1.5 um^2, occupying about 0.212% of the cell surface. They found an average packing density of intramembrane particles (connexons) or complementary pits of about 7,200 per um of nexal surface and extrapolated this as indicating that there may be up to 77,000 intercellular channels within the total complement of nexuses of a single muscle cell. In a further study, Gabella (1979) produced muscle hypertrophy in guinea pig ileum by experimental stenosis and found an increase in size and number of cells, with gap junctions larger than in control animals. However, while cells doubled their surface area, the number of nexuses per unit area remained unchanged.

As indicated above, gap junctions are widely distributed throughout the animal kingdom (Gilula, 1977). Indeed their absence in animal tissues is exceptional. They do not normally occur between circulating blood cells nor between mature skeletal muscle cells, where they have been rendered redundant by cell fusion into a syncytium, and do not occur between neurones and glial cells (Bennett and Goodenough, 1978). In all sites, they have a similar, if slightly variable, appearance as described above. Recently, studies on gap junctions or nexuses have involved techniques such as freeze fracture diffraction, negative staining and biochemical methods and little attention has been paid to older methods. The junctions were described first by routine electron microscopy but it was the work of Revel and Karnovsky (1967) that added much to our knowledge of junctional structures. They used the colloidal lanthanum tracer technique that permitted visualization of the gap junctional lattice in cardiac and liver tissue. Recently, a study of duodenal enterocytes compared basic aldehyde fixation with results using Malachite Green or lanthanum chloride (Leeson and Higgs, 1982). The results suggested that the addition of lanthanum to the fixative was valuable and recent work on lanthanum chloride as a marker for intercellular junctions in rat exocrine pancreas has been reported also (Leeson and Leeson, 1982). The purpose of the current study is to demonstrate the use of this lanthanum tracer technique in investigating gap junctions of smooth muscle in the muscularis of the rat duodenum.

MATERIALS AND METHODS

After anesthetization with ether, thoraxes of twelve young adult rats (50 to 120 days) were opened and the animals perfused via the left ventricle with a fixative containing glutaraldehyde, 3%, and formaldehyde, 3%, in 0.2M cacodylate buffer at pH 7.2, and 10mM with respect to lanthanum chloride (Sigma Chemical Company). A further six rats as controls were treated similarly except the basic fixative without lanthanum chloride was used. In all animals, the duodenum was removed, minced and placed in fresh fixative for two hours, washed in buffer,

dehydrated through graded ethanols, and infiltrated and embedded in an Epon-Araldite mixture, the tissue oriented as far as possible to permit either transverse or longitudinal sectioning. Ultrathin sections (silver grey) were cut on a Porter Blum II ultramicrotome and supported on 300 mesh grids. Sections were examined without further staining, or after staining either with alcoholic uranyl acetate for 10 minutes or lead citrate for three minutes, or both. Electron microscopy was performed with a Philips EM 200 at 60 KV or an EM 410 at 80 KV.

RESULTS

In all the material examined, no nexuses between muscle cells were seen in the outer longitudinal coat of the duodenal muscularis although nexuses were frequent in the inner circular layer. In control material, i.e. in material not exposed to lanthanum chloride, nexuses were seen with difficulty in unstained sections where generally they only were visible as 'linear' profiles, i.e. as pentalaminar structures. Where sectioned in planes other than perpendicular to the nexus, they proved very difficult to identify. In stained sections of control material, nexuses sectioned in all planes were identifiable in the circular layer, although the great majority was the linear profile. In lanthanum treated materials, nexuses were markedly electron dense and easily seen even in unstained sections, although staining enhanced contrast of the tissue components. Section staining in this material apparently caused no change or improvement in appearance or contrast of the nexus. However, most illustrations are from stained material as overall tissue contrast and appearance were enhanced.

Nexuses varied in appearance and extent, presumably largely due to the plane of section. Some appeared extensive, others extremely small. In many cells, no nexus was seen in the section while others showed such contacts with one or more adjacent cells. Frequently, more than one nexal contact was made with another cell and occasionally a nexus occurred between two processes of the same cell. Nexuses were seen in all areas of the cell, i.e. in the nuclear region as well as the cytoplasmic extensions of the cell, and were formed by contacts between cell bodies or cell processes. In form, they varied with the plane of section from linear (from straight to curved) to oblique to tangential, i.e. cut through the nexus parallel to its surface, the thickness or "width" of the resultant profile correspondingly increasing. As the plane of section varied, so did the morphology of the nexus and these appearances combined made possible a three-dimensional interpretation.

In control material at low magnification, smooth muscle cells of both layers of the muscularis generally showed a close packing with little intercellular material and very few other cells, mostly fibrocytes. Between the two layers, intercellular material was more prominent with collections of ganglion cells and nerves of the intermyenteric plexus frequently seen. Small nerve bundles and occasional ganglion cells were seen between smooth muscle cells in each layer, more frequently in the peripheral zone of the circular layer. The junction between the two muscle layers was clear, as was the junction between the submucosa and the inner circular layer. Intermediate and gap junctions between muscle cells of the circular layer were identifiable at low magnification. At higher magnifications, nexal contacts in the main appeared as linear profiles, varying in extent and location, i.e. some occurring between cell surfaces, other between one cell and a process from another cell, or between processes from adjacent cells. The pentalaminar appear-

ance of these linear profiles was seen clearly at high magnification, the central dense lamina in some being continuous, in others interrupted and, where cut slightly obliquely, with dense transverse lines. Oblique and tangential sections through nexuses in this material proved difficult to identify and little detail in them was discernable. In cell cytoplasm adjacent to nexuses, small vesicles or caveolae and mitochondria were seen frequently.

In lanthanum treated material at low magnification, nexuses were seen easily due to their enhanced electron density regardless of grid staining or not. As in control material, their number, form and location varied widely. In many cases, in the inner circular layer the majority of cells showed at least one nexus around its circumference and many cells showed more than one. In a few instances, a nexus was present between a smooth muscle cell and an element of nerve tissue. While nexuses were absent in the longitudinal layer, muscle cells there showed numerous subsarcolemmal caveolae, these caveolae also being present in cells of the circular layer but in fewer numbers. The frequency and clarity of visualization of nexuses with lanthanum was demonstrated also at medium power with several such contacts between adjacent cells and, frequently, contacts between three cells or their processes.

At higher magnifications of nexuses showing basically a linear or pentalaminar appearance, there was some variation in appearance. In thicker sections, or where lanthanum penetration had been extensive, the central electron-dense lamina appeared thichened to about 4 nm and complete, with regularly spaced dense transverse lines passing across the junction where the plane of section was slightly oblique. In thinner sections, or where lanthanum penetration was less dense, the central dense lamina appeared interrupted at regular intervals. Often, a nexus showed at one extremity the pentalaminar structure with, at the other, a pattern of transverse lines, sometimes grading to an apparent pattern of circular profiles, this presumably resultant upon the plane of section. In more tangential or 'en-face' sections, further detail was apparent. In such sections, the nexus showed a pattern of small circular, low density profiles of 8 to 9 nm diameter arranged in hexagonal array, with dense lanthanum deposit around and between these profiles. In a few instances, the circular profiles showed a small, central dense dot. These morphological features of the nexus were also visible in unstained but lanthanum treated material. Also, in just two micrographs, lanthanum in addition to infiltrating a nexus also filled one or more small vesicles that communicated by necks with the intercellular space.

DISCUSSION

The history of our knowledge of gap junctions is relatively short but their form, distribution and function have aroused considerable attention in recent years. A hexagonal packing of subunits associated with plasma membranes after permanganate and osmium tetroxide fixation was described by Robertson (1963) in electrical synapses of Mauthner cells, and a similar pattern in negatively stained isolated hepatic cell plasma membrane junctions was illustrated by Benedetti and Emmelot (1965). Revel and Karnovsky (1967) first described the use of lanthanum salts to demonstrate a hexagonal array of subunits in intercellular junctions of mouse heart and liver and their report includes illustrations remarkably similar to those of this study. It was the study by Revel and Karnovsky that first distinguished

between true tight junctions (zonulae occludentes) and gap junctions and they observed that the hexagonal lattice outlined by the tracer material lay within the 2 nm gap. Later, it was shown that the nexus of smooth muscle also showed a 2 nm gap and a hexagonal lattice demonstrable by colloidal lanthanum (Revel, Olsen and Karnovsky, 1967). While other workers have used this technique since that time, most recent studies of the smooth muscle nexus and gap junctions in other tissues have utilized freeze fracture and other techniques. The current study reaffirms the value of a simplified lanthanum technique. This is not to deny the value of other techniques that have clarified and extended our knowledge and understanding of the gap junction.

The morphological detail revealed by using lanthanum is much greater than that after routine fixation and staining. Indeed, in intestinal smooth muscle this study confirms that nexuses are not seen easily and that the great majority are of the 'linear', pentalaminar type. A tangential section of a nexus apparently is not recognizable as such although Robertson (1963) did demonstrate them in permanganate-fixed tissue. In comparison, after lanthanum treatment, nexuses are identified easily at low magnifications which facilitates studies of their number, size and distribution, and at higher magnifications, nexuses cut in all possible planes are visible and details of structure are seen, varying with the plane of section. Indeed, while studies of smooth muscle using freeze fracture have given much information (Fry et. al., 1977: Gabella and Blundell, 1979: Gabella, 1979), similar information has been obtained with the much simpler tracer technique. Other techniques provide different and, often, additional information. For example, Raviola et. al, (1980), studied gap junctions in several non-muscle cells with freeze fracture after rapid freezing and demonstrated that they are highly pleomorphic in the living state, this perhaps accounting for the variations in structure reported after chemical fixation. As Peracchia and Peracchia (1980) have pointed out, there now is evidence that intracellular cations, particularly free calcium, have a role in the mechanism of cell uncoupling whereby direct cell to cell communication via gap junctions is interrupted. They studied such junctions isolated from lens fibers and exposed to a pH of 6.5 or lower to establish the role of H^+ in this process. They found that the freeze fracture appearance of both control and low pH treated junctions was not altered by glutaraldehyde fixation and cryoprotective treatment as had been suggested previously (Heuser et. al., 1975) in a study where intact and isolated junctions were freeze fractured after rapid freezing. At low pH, Peracchia and Peracchia showed that particles (connexons) in the gap junction most often form orthagonal and rhombic arrays. Recently, in mouse liver gap junctions isolated with detergents and negatively stained with uranyl acetate, Baker et. al. (1983) used low-irradiation methods for recording and showed that substructural features of the junction are acutely sensitive to irradiation. Their method revealed, in the hexagonal junction lattice, skewed hexameric connexons, suggesting that the two hexagonal membrane arrays that form the junction may not be structurally identical. Thus, while obviously valuable, the lanthanum tracer technique has its limitations.

Studies that have reported on the size and number of gap junctions and the packing density of particles of the gap junction as seen in freeze fracture preparations (Fry et. al. 1977; Gabella, 1979; Gabella and Blundell, 1979) have shown some variation, perhaps due to variation in cell and species types, but variations also occur with physiological state. In guinea pig ileum, nexuses of the circular

muscle layer occupy 0.212% of the cell surface and in each cell there may be up to 77,000 intercellular channels in the total nexus complement (Gabella and Blundell, 1979). After the production of hypertrophy following experimental stenosis, gap junctions increase in size and occupy 0.49% of the cell surface (Gabella, 1979). Differences in gap junction size between cultivated normal and hypertensive smooth muscle cells of the rat aorta have been studied by freeze fracture (Grünwald et. al, 1982). These investigators found that the total surface area of plasma membrane occupied by gap junctions was much smaller in normal than in hypertensive smooth muscle and concluded that the results indicate an intense metabolic communication between cells in the hypertensive smooth muscle cell cultures. Other cell types show changes in gap junctions with physiological state. For example, exogenous estrogen stimulation promotes gap junction growth indirectly in oravian granulosa cells while exogenous FSH stimulation directly amplifies the developmental sequence of gap junction growth and turnover (Burghardt and Matheson, 1982). Several studies have been performed on uterine smooth muscle. Reports that bilateral section of the pelvic parasympathetic nerves in the rat block parturition led to a study of myometrial gap junctions (Burden et. al, 1979). These investigators found a significant increase in the number of gap junctions by day 23 in the animals after pelvic neurectomy and concluded that the parturition block is not attributable to failure of gap junction formation in smooth muscle cells. Garfield and Hayashi (1980) suggested that gap junctions between myometrial cells in nonpregnant women may form in response to physiological and pathological stimuli such as the production of prostaglandins and that the presence of these intercellular contacts may result in contractions of the uterus. In a subsequent study (Garfield and Hayashi, 1981), the same investigators studied myometrial tissue in various stages of labor and found increasing gap junctions with increased cervical dilatation or increased frequency of uterine contractions. They proposed that gap junction formation may be stimulated by some physiologic change and that the appearance of gap junctions may terminate pregnancy by resulting in coordinated, synchronized muscle activity and dilation of the cervix. In a study in guinea pigs, gap junctions in myometruim were found to increase at parturition (Garfield et. al., 1982), suggesting that an increase in myometrial gap junction area is associated with, and may be essential for, parturition. In chicken gizzard smooth muscle over several developmental stages, La Manita and Shafiq (1982) using the freeze fracture technique found not only an increase in intramembranous particles per gap junction but also an increase in particle density. Thus, in several tissues, gap junctions show changes with age, and with physiological and pathological state. Such studies of size, number, packing density could be undertaken using the lanthanum tracer technique, although one report (Fry, et. al., 1977) has suggested that small nexuses may not be recognizable as such in thin sections.

As stated earlier, many studies of the nexuses of intestinal smooth muscle have been concerned with electrical coupling (Daniel et. al., 1976) and this study has confirmed previous reports of the sparsity or absence of nexuses between muscle cells of the outer longitudinal layer (Fry et. al, 1977; Gabella and Blundell, 1979: Henderson et. al, 1971; Daniel et. al, 1966; Gabella, 1979). Among others, Gabella (1979) has suggested that electrical coupling is achieved by a mechanism not involving gap junctions. In an interesting study of the connective tissue of longitudinal and circular muscle of the rat small intestine, Taylor et. al, (1977)

observed electrotonic spread of applied potentials between the muscle layers with no rectification, and found nexal junctions between muscle cells of both layers. Since connective tissue can serve for electrical conduction between cultured heart cells and since electrical properties of intestinal muscle permit transmission with low degrees of coupling, they suggested that interstitial cells and fibrocytes may electrically couple both muscle layers in the rat intestine. This is an interesting hypothesis that merits further study.

CONCLUDING REMARKS

This study using a simplified tracer technique has shown that much useful information can be obtained concerning the location, number, size and general distribution of nexuses in intestinal smooth muscle, and some information about the structure of the nexus in respect of particle (connexon) size and packing. The results have been discussed in relation to current knowledge of the nexus/gap junction, its morphology, functions and variations that have been reported with changes in methodology and physiologic and pathologic state. While our knowledge of the gap junction has improved steadily over the past decade and a half, much work remains to be done, for example, even in the area of the correlation between morphological studies in intestinal muscle and the apparent species variation in relation to electrical coupling. It is suggested that in future studies, the use of simple tracer techniques should not be ignored as they still can facilitate such investigations.

DESCRIPTION OF ILLUSTRATIONS

Plate I

Fig. 1. Muscle fibers (L) of the outer longitudinal layer of the muscularis of rat duodenum are seen at lower left and show no nexuses in this section treated with lanthanum and stained with lead citrate and uranyl acetate. Muscle fibers of the inner circular layer occupy the remainder of the field and show numerous, electron-dense nexuses (arrows). Also seen are a small nerve (N) and blood vessel (V). x 3,500.

Fig. 2. An unstained section from a control (non-lanthanum) rat showing a process (P) of a muscle cell of the circular layer forming nexuses with two other cells (lower left, top right). Both nexuses show a pentalaminar structure with deficiencies in the central dense lamina (arrowheads) indicating connexons. x 115,000.

Fig. 3. An extensive nexus from a lanthanum treated, unstained section showing the pentalaminar structure cut transversely, with regular deficiencies in the central dense lamina, but showing regular, transverse linear densities (arrows) where cut slightly obliquely, these indicating lanthanum penetration between connexons. x 105,000.

Plate II

Fig. 4. Portions of three muscle fibers (1, 2, 3) are seen with linear nexal contacts (top and bottom) densely stained by lanthanum and with thick, continuous central dense laminae. The central nexus involves all three cells and is sectioned obliquely with little detailed structure apparent. The section was block-stained. x 52,000.

Fig. 5. A nexus here is cut transversely, from lanthanum and double stained material. The pentalaminar structure is seen with a dense central lamina, mainly continuous but with some interruptions (arrow). x 85,000.

Fig. 6. This section, from block-stained, lanthanum treated material, shows two nexuses between three muscle cells, the one on the left cut transversely and showing the pentalaminar structure, the one on the right cut obliquely and showing a pattern of electron lucid, small circular profiles (connexons) (arrow) in electron-dense material. x 74,000.

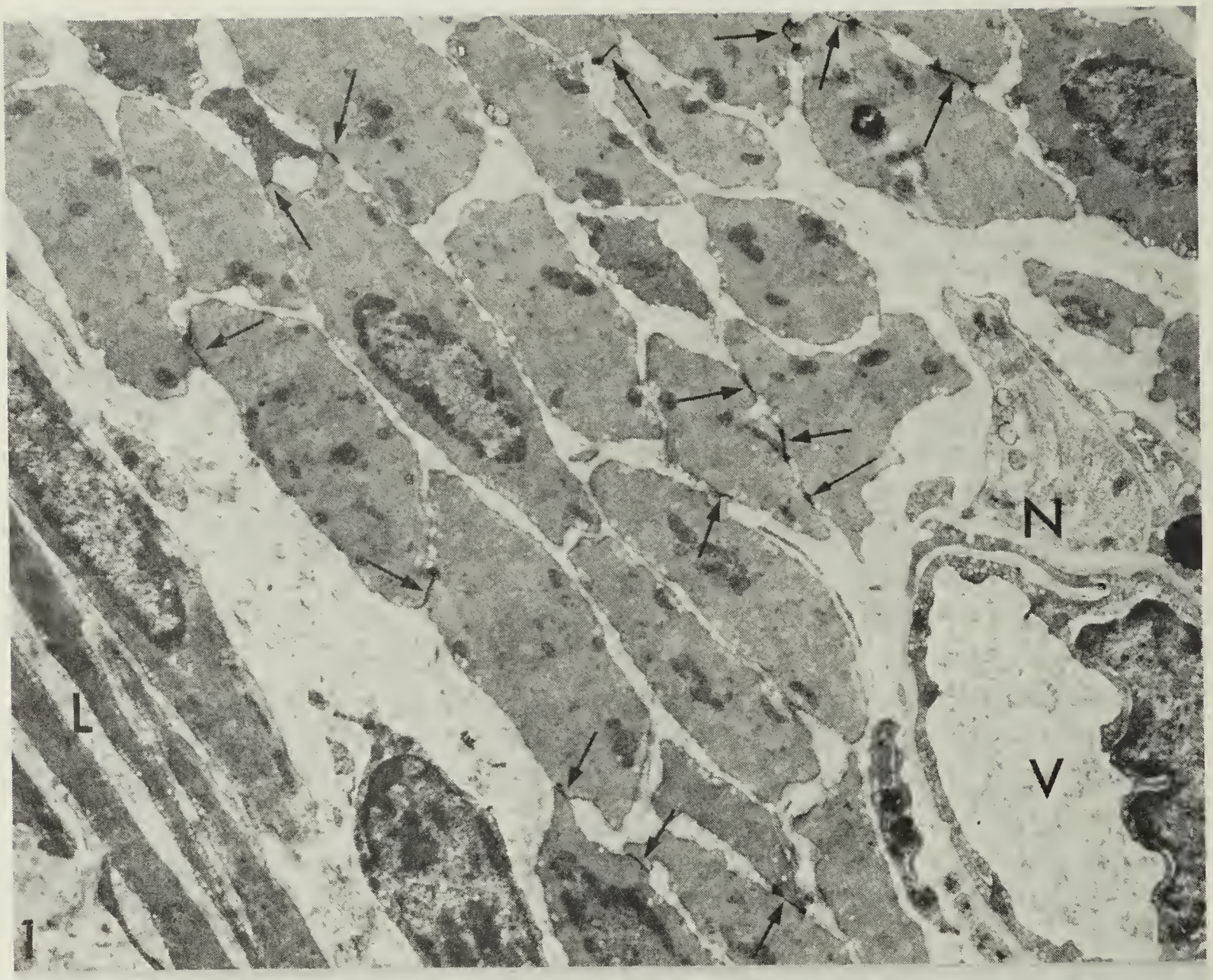
Fig. 7. This S-shaped nexus is from unstained, lanthanum treated material. In this 'en-face', tangential section through the center of the nexus a regular hexagonal pattern of electron lucid particles or connexons is seen, lying in electron dense material (arrows). Centrally, where the plane of section is oblique, this pattern is not seen (asterisk). x 115,000.

Plate III

Fig. 8. In this unstained but lanthanum treated material a muscle cell (M) makes nexal contacts (left) with two other processes of muscle cells (P), here sectioned nearly transversely but showing a thick central dense lamina. At right, a further nexus (arrow) is cut 'en-face' and shows a regular hexagonal pattern of electron lucid connexons with dense material surrounding them. x 74,000.

Fig. 9. Similar to Figure 8, but double stained and showing a nexus 'en-face' with pale particles or connexons in hexagonal array, some with a small, central dark dot (arrowheads) indicating the central channel of the connexon. x 210,000.

Fig. 10. A nexus between two muscle cells is seen, showing at top and bottom the pentalaminar structure with a continuous, dense central lamina but centrally the two plasmalemmae separate and in this area two small vesicles or caveolae (arrows) show continuity with the extracellular space and contain dense material. The material was double stained and lanthanum treated. x 115,000.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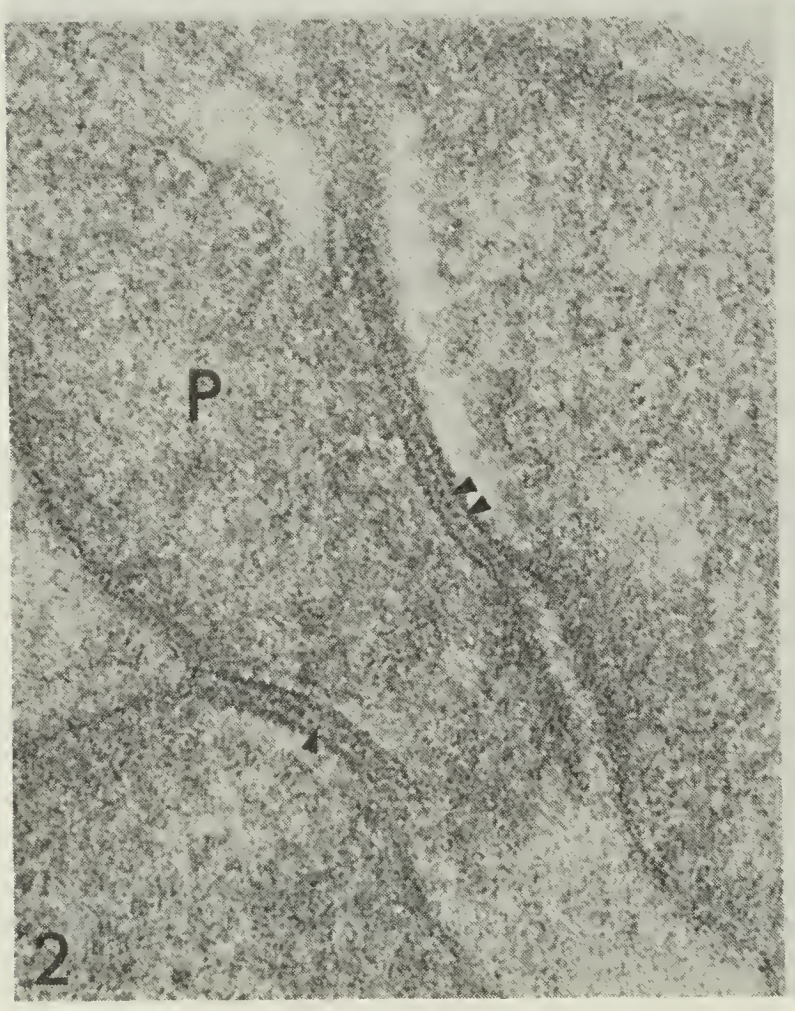
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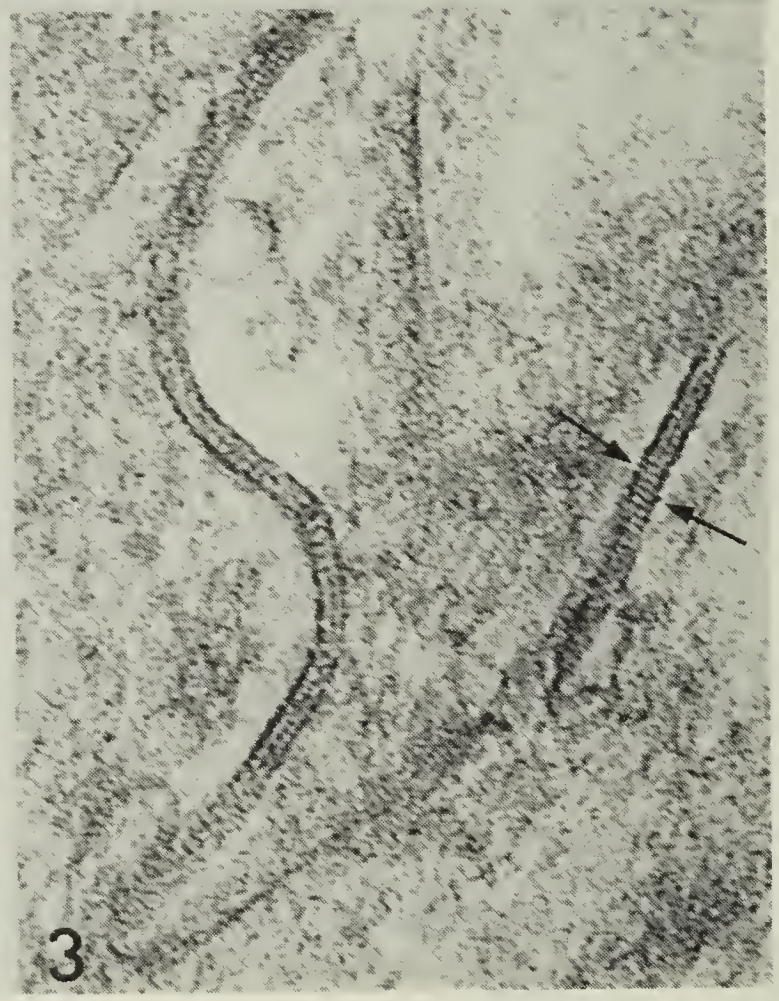
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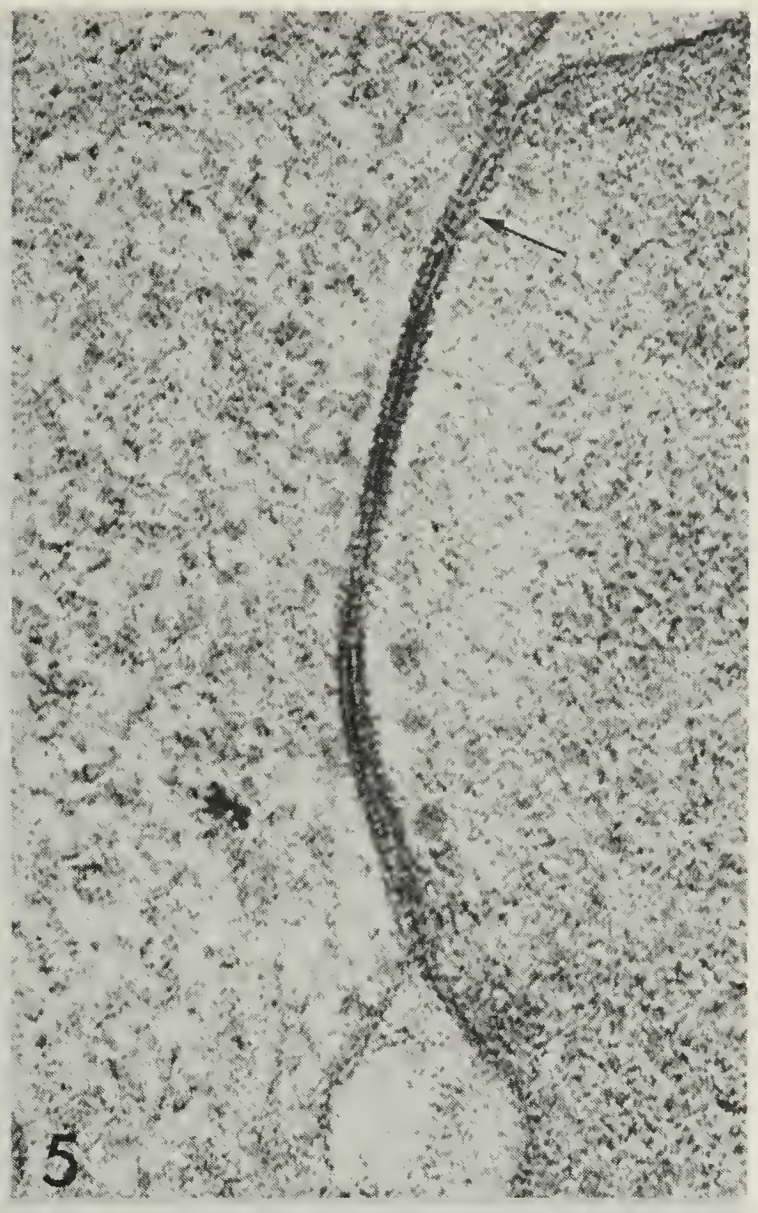
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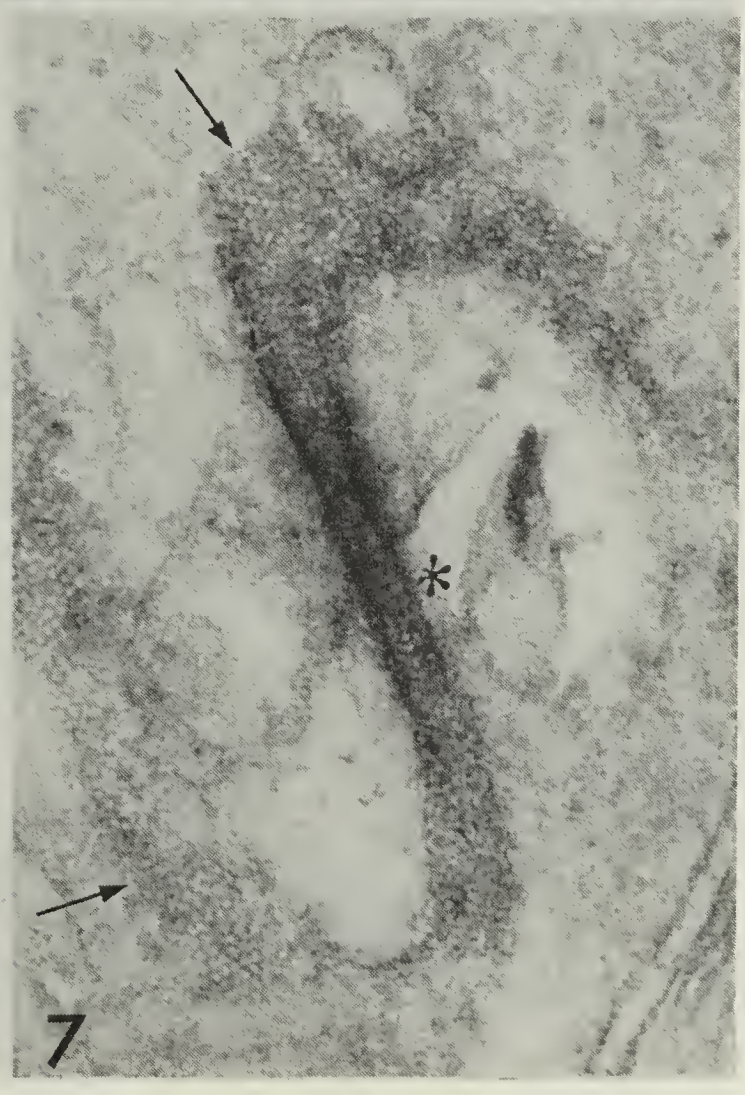
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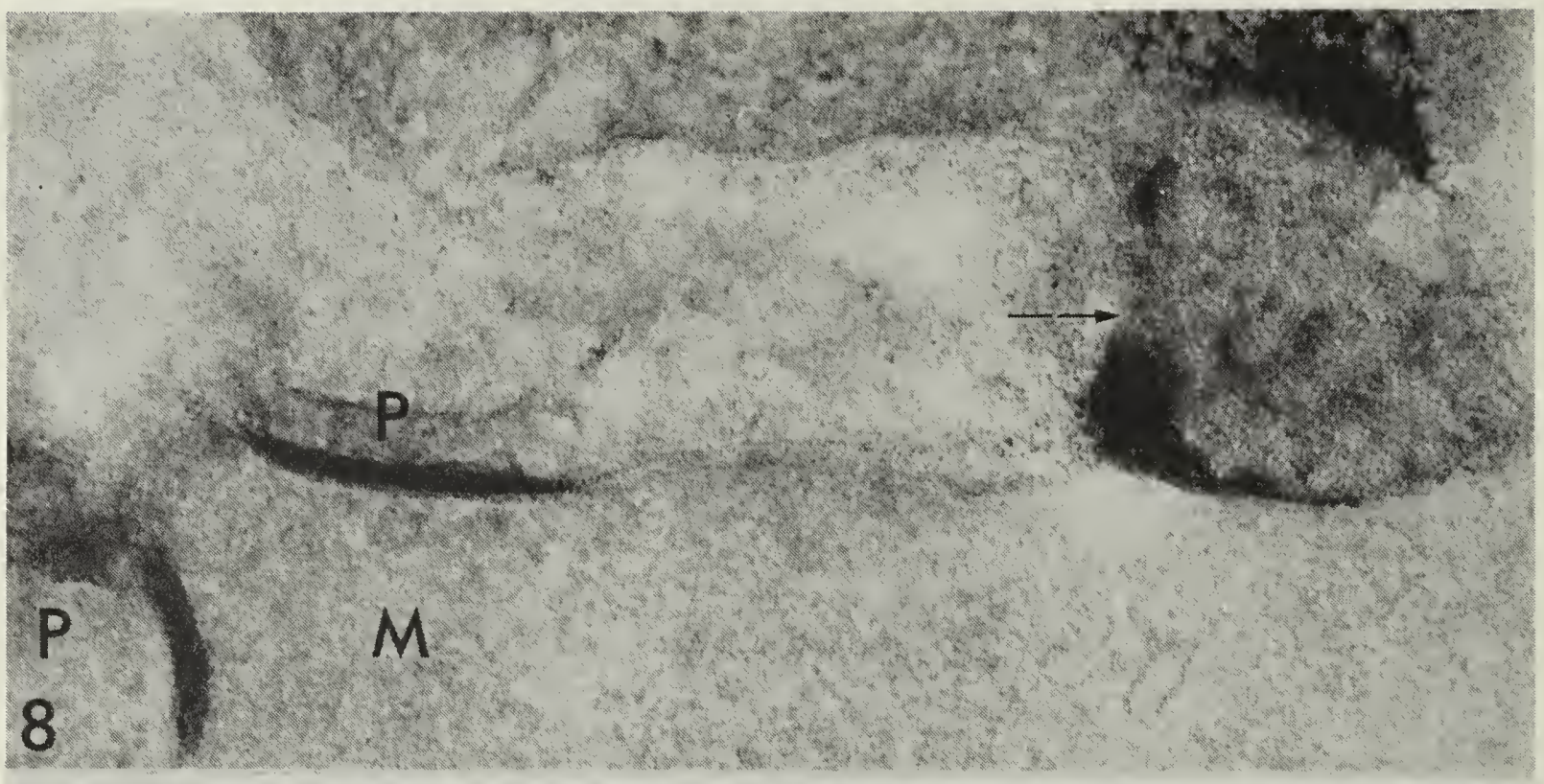
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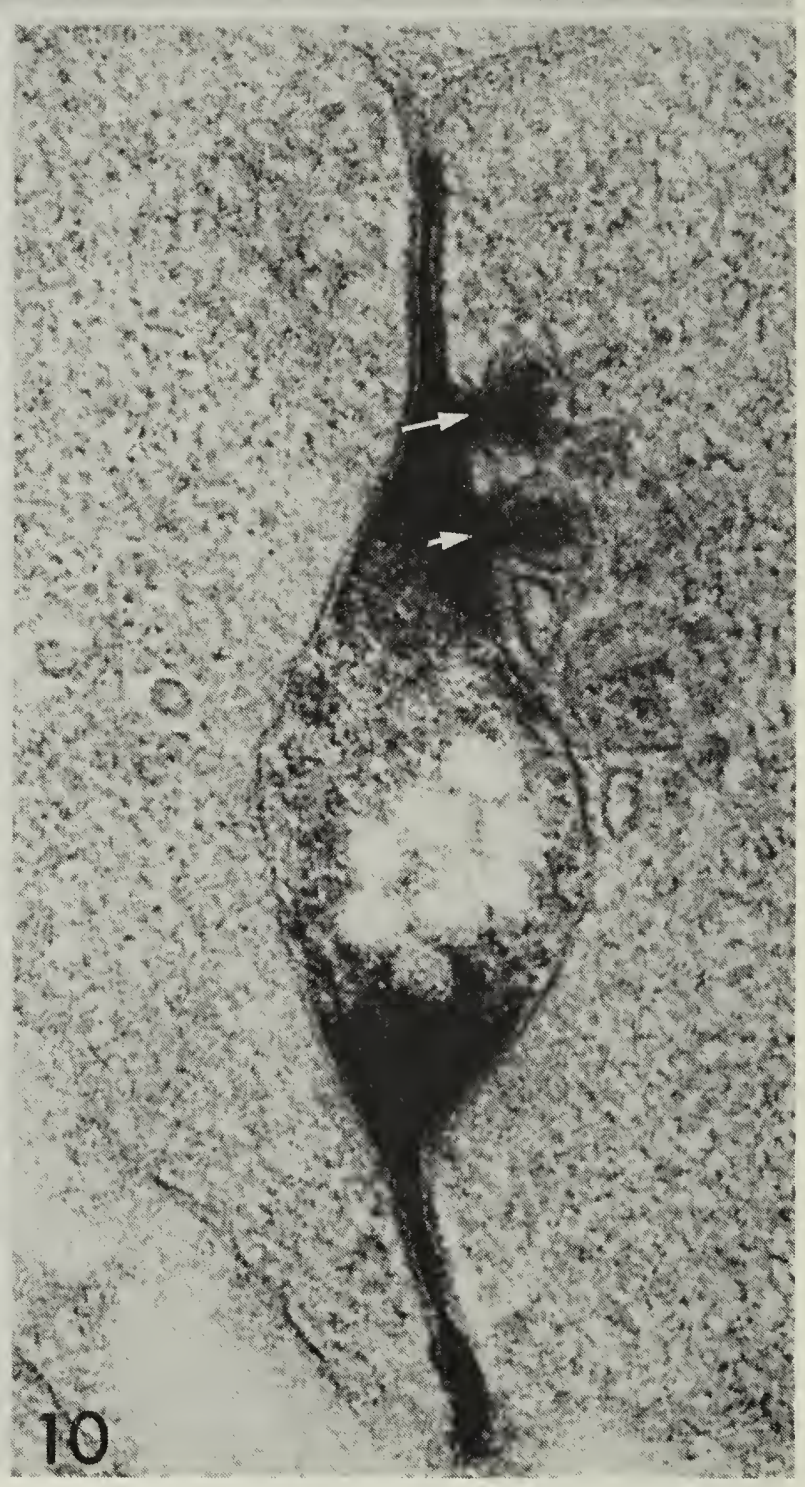
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Effects of Trifluoperazine on the Activation of Cilia by 5-Hydroxytryptamine in *Mytilus edulis*

Akira Murakami
Zoological Institute
Faculty of Science
University of Tokyo, Hongo
Tokyo 113, Japan

ABSTRACT

1. The effects of trifluoperazine (TFP), a phenothiazine inhibitor of calmodulin, were studies on the activation of ciliary beat by 5-hydroxytryptamine (5-HT) in the lateral cells of the gill in *Mytilus edulis*. 2. The ciliary activation was totally blocked by 20 μm TFP. The inhibition was Ca-dependent. 3. At a concentration of 5-10 μm, TFP induced repetitive ciliary arrest responses of the lateral cells. 4. Deciliation was observed in the medium containing TFP at a concentration of 20 μm and above. 5. These results suggest that TFP increased the cytoplasmic Ca^{2+} of the ciliated cells and induced the ciliary arrest. Other possible mechanisms of the inhibition are discussed.

INTRODUCTION

Calmodulin (CaM) or calmodulin-like proteins mediate various kinds of regulatory activities in a wide range of cell types (Means and Dedman, 1980; Cheung, 1980; Means, Tash and Chafouleas, 1982). Within a single cell, CaM may control more than one type of response. In a protozoan cell such as *Paramecium* or *Tetrahymena*, for example, CaM appears to regulate such activities as the activation of the Ca^{2+} pump in the membrane, the ciliary reversal and the trichocyst discharge etc. (Satir, Garofalo, Gilligan and Maihle, 1980; Garofalo, Gilligan and Satir, 1983; Suzuki, Ohnishi, Hirabayashi and Watanabe, 1982).

In the gill of the mussel, the branchial nerve seems to control at least three different responses of the same cilia. Thus, repetitive stimulation given to the nerve at 2-5 pulses$\cdot s^{-1}$ increases the beat frequency of the lateral cilia. For this effect, 5-hydroxytryptamine (5-HT) is a putative transmitter substance released from the nerve terminals (Aiello, 1974). It has been reported that stimulation of the same

nerve at 25-50 pulses·s^{-1} caused a gradual decrease of the ciliary beat frequency lasting for 10 min or more (Paparo and Aiello, 1970). On the other hand, a single electric pulse given to the branchial nerve brings about an abrupt stoppage of the same cilia (Takahashi and Murakami, 1968). The ciliary arrest of the same type is induced by various stimuli that depolarize the lateral cell (Takahashi and Tsuchiya, 1971; Murakami and Takahashi, 1975a; Saimi, Murakami and Takahashi, 1983a; 1983b). The arrest response is Ca-dependent and most likely regulated by the level of cytoplasmic Ca^{2+} (Tsuchiya and Takahashi, 1972; Tsuchiya, 1977; Walter and Satir, 1978).

In detergent-treated models of the lateral cells the ciliary arrest is induced by an addition of Ca^{2+} to the medium (Tsuchiya, 1977; Walter and Satir, 1978) and the arrest is inhibited by a phenothiazine, trifluoperazine (TFP), which antagonizes the action of CaM (Levin and Weiss, 1976). It has been suggested that CaM may be involved in the regulatory mechanism underlying the arrest of the lateral cilia (Reed, Lebduska and Satir, 1982). In the gill of *Aequipecten irradians*, however, it has been reported that most of the CaM in cilia is localized not in the ciliary axonemes but in the detergent-solubilized fraction of the membrane-matrix and also that the effect of TFP on the ciliary arrest is non specific (Stommel, Stephens, Masure and Head, 1982). It seems likely that the principal function of CaM in some ciliated cells may be related to the activities of the membrane such as the Ca^{2+} transport by the Ca^{2+} pump (Vincenzi and Hinds, 1980; Stommel *et al*, 1982) and the formation of adenosine 3′, 5′-cyclic monophosphate (cAMP) by adenylate cyclase (Bradham and Cheung, 1980; Means *et al*, 1982). It is interesting in this connection that cAMP has been suggested to be involved in the activation of some cilia and flagella including the lateral cilia of *Mytilus* (Malanga and Poll, 1979; Means *et al*, 1982; Morisawa and Okuno, 1982; Murakami, 1983).

We report here that TFP strongly inhibits the ciliary activation of the lateral cilia of *Mytilus* by 5-HT. The inhibition is Ca-dependent. The results suggest that CaM may play multiple roles in the lateral cells. The possible role of CaM in the regulatory systems of the ciliary motility is discussed in relation to the effect of TFP on the ciliated cells.

MATERIALS AND METHODS

Most of the experiments were carried out on the lateral cilia on the gill filaments of *Mytilus edulis*. A few observations were made on the latero-frontal cilia and the cilia in the ciliary junctions between the gill-filaments (ciliary pads). The specimens were collected near the Misaki Marine Biological Station, brought to the laboratory in Tokyo and kept in an aquarium of circulating natural sea water (water temperature, 19°C).

A gill-filament was isolated from the descending lamella of a demibranch. The isolated filament was split into two lateral halves along its entire length to expose the basal side of the ciliated epithelium. One of the half gill-filament preparations was then placed straight in a perfusion vessel with its lateral side facing downward. The vessel had a capacity of about 0.4 ml. It was continuously perfused with artificial sea water (ASW, see below) at a rate of 1.6 ml·min^{-1}.

In order to monitor the frequency of the ciliary beat photoelectrically (Takahashi and Tsuchiya, 1971), the microscopic image of the cilia was projected onto a photomultiplier tube (Toshiba, MS-9S) through a pinhole in a screen placed in front of the tube. The photoelectric current was amplified and displayed on a

cathode ray oscilloscope. The frequency of its fluctuations caused by the ciliary beat was counted directly on the screen of the oscilloscope or by means of an electronic counter (Takahashi and Tsuchiya, 1971).

The ASW used had the following composition (in mM): MaCl, 434; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 53; N-(2-hydroxyethyl)-piperazine-N′-2-ethane sulfonic acid (HEPES), 10 (pH 7.5). To obtain the Ca-free ASW, the $CaCl_2$ in the ASW was replaced with an osmotically equivalent amount of NaCl. The Ca-free ASW containing 1 mM ethyleneglycol-bis(β-aminoethyl ether)N, N′-tetraacetic acid (EGTA) (EGTA-ASW) was usually applied after washing the preparation with Ca-free ASW. ASW containing 10^{-7} M 5-HT was used to stimulate the cilia to beat.

All the experiments were carried out at room temperature (22°-24°C).

RESULTS

Unless stimulated with 5-HT, the lateral cilia on the half gill-filament preparation were usually quiescent. An addition of 5-HT to the medium stimulated the cilia to beat (Aiello, 1974). The threshold concentration of 5-HT was between 10^{-10} and 10^{-9} M. The cilia started beating within 20 s after the onset of perfusion with 10^{-7} M 5-HT. The beat frequency of the activated cilia reached a plateau after a min or so. The maximal frequency was about 25 s^{-1} (range, 21.4-32.5 s^{-1}).

The activation of ciliary beat by 5-HT was inhibited by TFP. The preparation was immersed in the medium containing TFP at various concentrations (5, 10, 15, 20, 25 μm) prior to the application of 5-HT. The test solution for the ciliary activation contained the same concentration of TFP in addition to 10^{-7} M 5-HT. The number of cells activated by 5-HT decreased as the concentration of TFP was increased (Fig. 1). The maximal beat frequencies of the cilia activated by 5-HT are plotted against the concentration of TFP in Fig. 2. The ciliary beat was irregular in the medium containing 5 or 10 μm TFP; transient and local arrests of the cilia were often observed. In 15 μm TFP, the frequency of the activated beat was reduced to about a half. No ciliary beat was induced by 5-HT in the presence of 20 μm TFP.

Observations were made on the changes of ciliary activity following an addition of 20 μm TFP to the medium containing 10^{-7} M 5-HT in which the lateral cilia had been activated. Local and transient arrest responses and a synchronous, rather than metachronous, beating along the length of the gill filament started about 1.5 min after the addition of TFP. The beat frequency decreased and the cilia finally stopped within 3 min. A further exposure to 20 μm TFP induced a detachment of up to 30 percent of the lateral cilia.

Exposure to 10^{-7} M 5-HT in the presence of 25 μm TFP failed to activate the lateral cilia. After about 5 min, 40 percent of the lateral cilia and more than 80 percent of the latero-frontal cilia were detached. The basal part of the latero-frontal cilia remained in the preparations after the deciliation. The detachment site of the lateral cilia could not be seen. The lateral cilia that were left attached after the treatment with 20 μm TFP for 10 min scarcely recovered the beat when washed with normal ASW and then stimulated with 10^{-7} M 5-HT. On the other hand, more than 90 percent of the lateral cilia recovered after a 10 min exposure to 15 μm TFP. No deciliation was observed in 15 μm TFP.

There were obvious differences in the response to TFP among the different kinds of cilia on the same preparation. The latero-frontals seemed less sensitive to

TFP than the laterals, so far as the motility was concerned. The reverse was true for the deciliation. The cilia of the ciliary pads (interfilamentar junctions) kept beating apparently normally in 20 μm TFP. The medium containing 100 μm TFP killed all the cells in the preparations within a min.

Chlorpromazine (CPZ) was less effective than TFP. An addition of 50 μm CPZ to the medium containing 10^{-7} M 5-HT induced the lateral cilia to beat irregularly; transient arrest response and synchronized beating appeared repeatedly. The maximal beat frequency of the cilia was about 60 percent of the control without CPZ.

The inhibitory effect of TFP on the ciliary activation by 5-HT was Ca-dependent. In Fig. 3 ciliary beat frequencies are plotted against the time after the addition of 10^{-7} M 5-HT. It has been known that the lateral cilia can be activated by 5-HT without extracellular Ca^{2+}, or in EGTA-ASW (Murakami and Takahashi, 1975b). In EGTA-ASW containing 20 μm TFP more than 80 percent of the lateral cells were activated by 5-HT. The maximal frequency of the activated cilia was 60-75 percent of the control without TFP (Fig. 3). No deciliation was observed in EGTA-ASW containing 20 μm TFP.

DISCUSSION

In the present study we found that TFP strongly inhibits the activation of the lateral cilia by 5-HT (Figs. 1 and 2). The inhibitory action of TFP in Ca-dependent (Fig. 3). TFP is phenothiazine known to inhibit the action of CaM (Levin and Weiss, 1976; Satir *et al*, 1980). Multifunctional actions of CaM on protozoan cells have been reported several times (Satir *et al*, 1980; Suzuki, Ohnishi, Hirabayashi and Watanabe, 1982). On the other hand, the regulation of the ciliary arrest response is the only function of CaM so far investigated in the ciliated cells in the gill of the bivalves (Reed, Lubduska and Satir, 1982; Stommel *et al*, 1982). If TFP inhibits the ciliary arrest, the ciliary beat would not be interrupted by the ciliary arrest in the presence of TFP. The present results were rather reverse in appearance: the ciliary beat was arrested in the medium containing 5-HT and TFP.

We may interpret the results in three ways: First, TFP may directly inhibit the mechanism by which 5-HT stimulates the ciliary beat. CaM has been known as a regulator of adenylate cyclase (Bradham and Cheung, 1980). It has been suggested that cAMP is involved in the regulatory mechanism of flagellar and ciliary motility (Malanga and Poll, 1979; Means *et al*, 1982; Morisawa and Okuno, 1982; Murakami, 1983). Second, TFP may inhibit the Ca^{2+} pump in the membrane and thus increases the level of the intracellular Ca^{2+}. It has been reported that an increase in the concentration of Ca^{2+} over 10^{-6} M inhibits the activity of adenylate cyclase irrespective of the presence or absence of TFP (Potter, Piascik, Wisler, Robertson and Johnson, 1980). The 5-HT-induced activation of cilia is possibly inhibited by an increase in Ca^{2+} through a block of adenylate cyclase. Third, if TFP induces an increase in Ca^{2+} and if TFP does not inhibit the mechanism of ciliary arrest under the conditions of the present experiment, the ciliary beat would be stopped by the Ca-dependent arrest response, even though the mechanism of ciliary activation by 5-HT is intact. It has been repeatedly demonstrated that the ciliary arrest response of the lateral cilia can be induced in the presence of 5-HT (Takahashi and Murakami, 1968; Saimi *et al*, 1983a; 1983b).

The deciliation induced by 20 or 25 μm TFP may indicate an increase in the intracellular Ca^{2+} (Satir *et al*, 1980). Spontaneous repetitions of the ciliary arrests

were observed in the solution containing 5 or 10 μm TFP, or 50 μm CPZ which is another phenothiazine inhibitor of CaM (Levin and Weiss, 1976). Ionophores such as X537A and A23187 induce similar repetitions of the ciliary arrest (Murakami and Takahashi, 1975b; Satir, 1975; Walter and Satir, 1978). Deciliation and repetitive arrests have also been induced in the gill cilia of *Aequipecten irradians* by TFP though at a higher concentration (more than 200 μm, Stommel *et al*, 1982).

The present results seem to suggest the third possibility mentioned above: that is, a transient increase in the Ca^{2+} level induces the ciliary arrest, at least in the medium containing less than 15 μm TFP. It remains to be determined, however, whether the level of cAMP is affected under the present experimental conditions; the triggering of the ciliary arrest may occur in parallel with the inhibition of the 5-HT-activation of cilia. It would be an interesting hypothesis that both CaM and cAMP are involved in the regulation of the activity of the lateral cilia. Mobilization of intracellular Ca^{2+} (Paparo and Murphy, 1975) and an increase in the level of cAMP (Malanga and Poll, 1979) have been reported to occur when the gill is stimulated by 5-HT.

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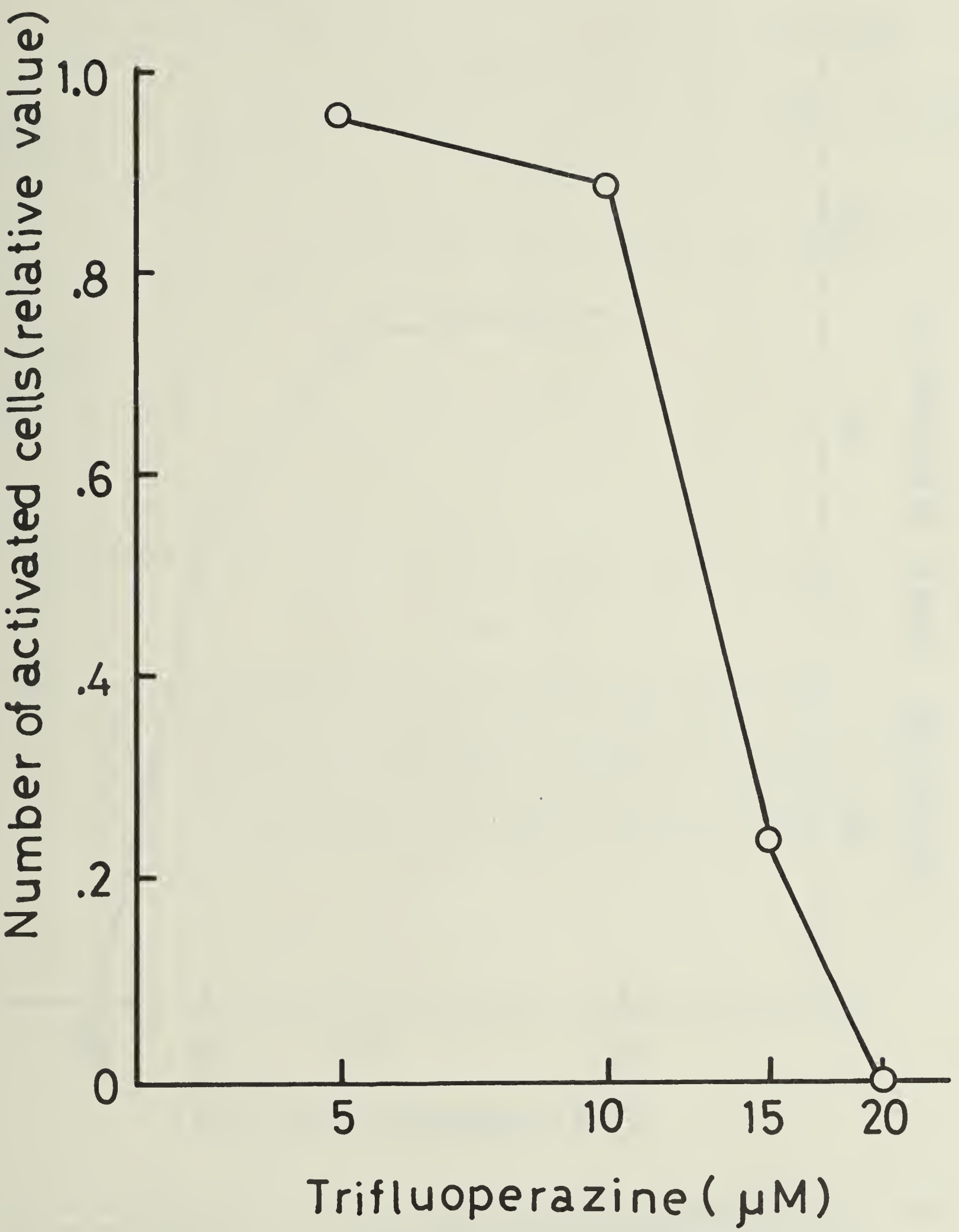

Fig. 1. Effect of trifluoperazine (TFP) on the number of lateral cells activated by 10^{-7} M 5-HT. The numbers of active and inactive cells were either directly counted when they were limited, or estimated by the length of the active and inactive regions along the gill axis as fractions of the total (ca. 50 cells or ca. 850 μm) in the microscopic field. The preparations were immersed in the medium containing TFP for 3 to 4 min prior to the perfusion with the medium containing 5-HT as well as TFP at the same concentration. All the cells were activated to a plateau within a min in the control medium without TFP. The estimations were made 3 to 5 min after the onset of the perfusion. Each point is the mean value of measurements on 3 to 7 preparations from different mussels.

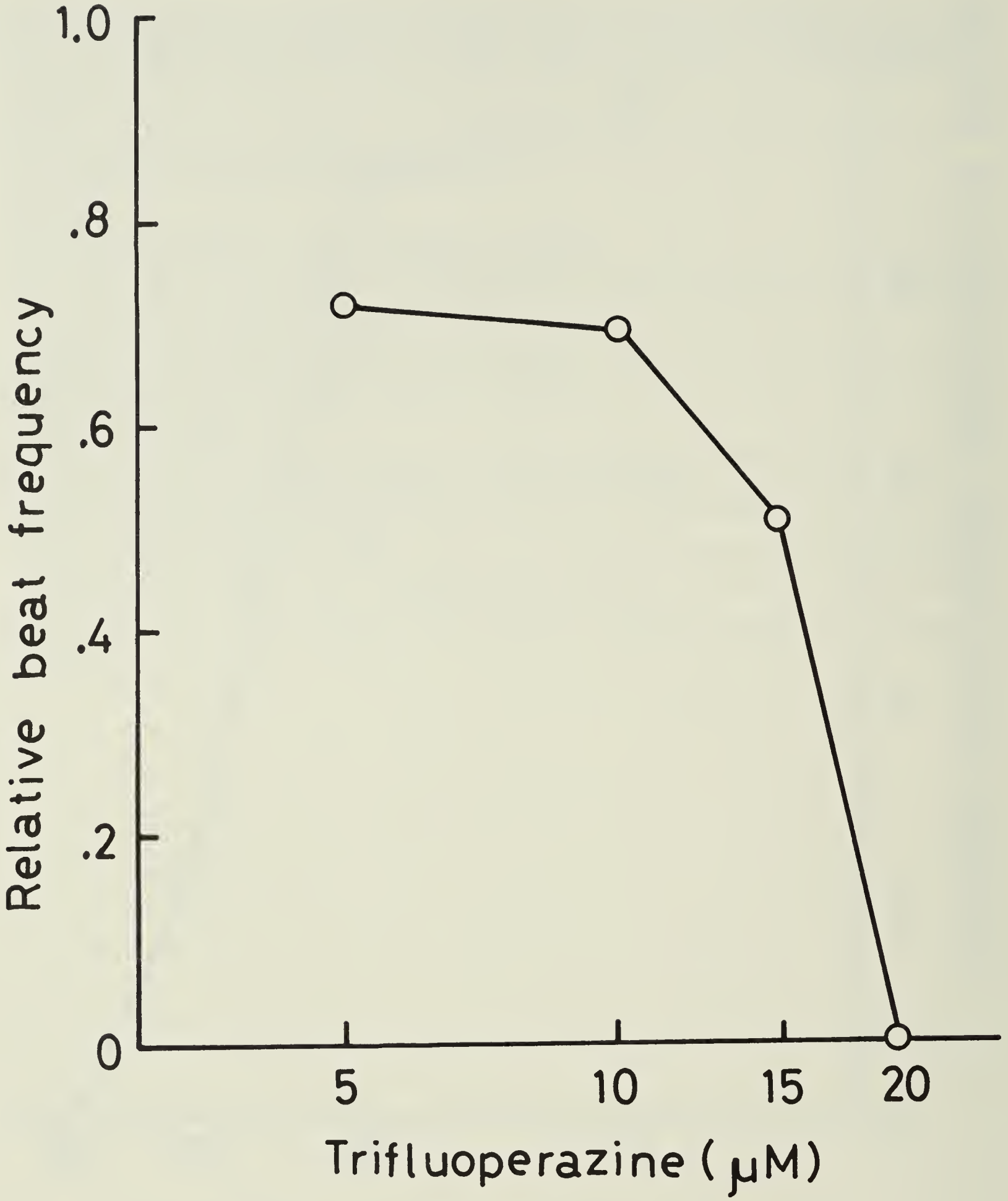

Fig. 2. Effect of trifluoperazine (TFP) on the maximal frequency of the lateral cilia activated by 10^{-7} M 5-HT. The beat frequency of the plateau observed in each solution is normalized with respect to that of the control in the normal artificial sea water without TFP (range 21.4-32.5 s^{-1}). The estimations were made 3 to 5 min after the onset of the perfusion. Each point is the mean of the values on 3 to 7 preparations from different mussels.

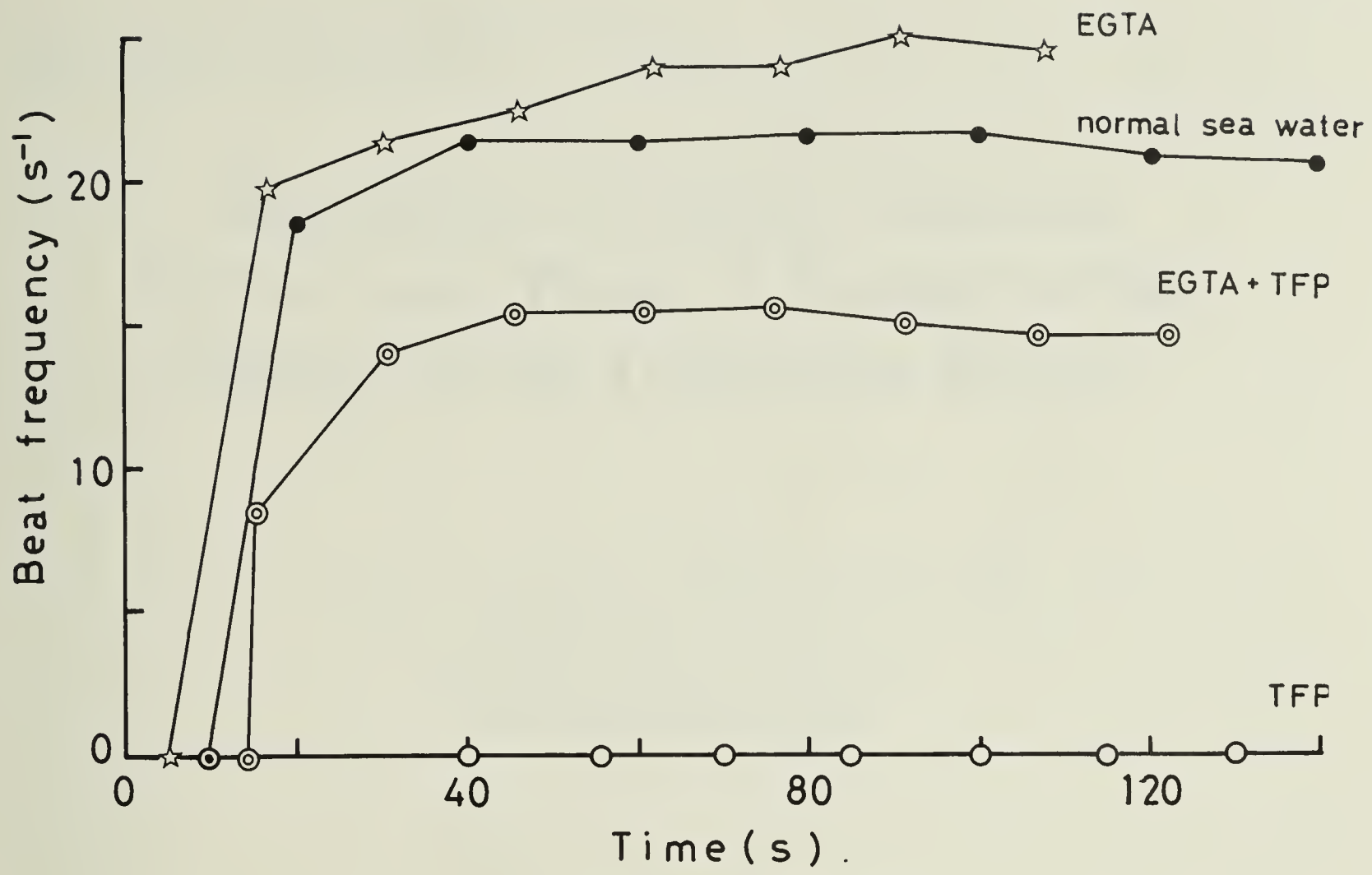

Fig. 3. Ca-dependence of the effect of trifluoperazine (TFP) on the ciliary activation by 5-HT. A typical example of a series of experiments; beat frequencies of the lateral cilia are plotted against the time in s after the onset of the perfusion with the 5-HT solutions under 4 different conditions. The cilia were activated by 5-HT (10^{-7} M) in the Ca-free artificial sea water containing 1 mM EGTA and 20 μm TFP (EGTA + TFP; double circles), while 20 μm TFP totally blocked the action of 5-HT when added to the normal (10 mM Ca) artificial sea water (TFP; open circles). The controls in the normal artificial sea water (normal sea water; solid circles) and in the Ca-free artificial sea water containing 1 mM EGTA (EGTA; stars) are also shown in the figure.

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Regulation of Platelet Function in the Acute Phase: A Review of the Biology of the C-Reactive Protein

Barry A. Fiedel
Department of Immunology/Microbiology
Rush Medical Center
1753 West Congress Parkway
Chicago, Illinois 60612

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ABSTRACT

Earlier investigations had demonstrated that CRP inhibited platelet aggregation and release reactions, activation of platelet factor 3 and platelet-dependent clot retraction, and suggested that CRP exerted these effects by interfering with an aspect of prostaglandin metabolism. Recent studies pointed to a role for an accessory molecule in the platelet inhibition mediated by CRP, and led to the conclusion that the platelet inhibitory properties of CRP result entirely from its association with a low-molecular weight factor (LMF) of approximately 8,300-12,500 daltons. The removal of LMF resulted in a CRP which could be thermally modified (H-CRP) to induce platelet aggregation/secretion in isolated systems and enhance the platelet activation in plasma induced by ADP, collagen or arachidonic acid. The activation of platelets by H-CRP was sensitive to nonsteroidal anti-inflammatory drugs and metabolic inhibitors as well as the ADP-removing enzyme system creatine phosphate/creatine phosphokinase. Thin-layer chromato-

graphic (TLC) analysis of prostanoate end-products following platelet activation with H-CRP revealed the formation of thromboxane A_2, an important endogenous platelet activator and contractor of vascular tissue; bioassay on rabbit aorta strips of supernatants obtained from platelets undergoing challenge with H-CRP supported the TLC analysis. Complexes formed between CRP and certain of its polycationic ligands were found to share platelet activating properties with H-CRP. These data imply an agonist role for CRP in platelet physiology, suggest that the interaction of modified CRP with the platelet at sites of vascular damage could have pathological significance, and support the concept that CRP can initiate biological activities similar to those mediated by immunoglobulin. In experiments directed to determine whether CRP and IgG might share a receptor on the platelet membrane, neither plasmin nor phospholipase C altered the platelet response to aggregate CRP (H-CRP) or aggregate IgG (AHGG), although these reagents enhanced the platelet expression to acid-soluble collagen (ASC). Conversely, chymotrypsin treatment of platelets resulted in an elevated response to each H-CRP and AHGG, but not to ASC. These data suggested that the H-CRP and AHGG platelet receptors share characteristics which contrast with those of the receptor for collagen. However, monomeric IgG, which can bind with the platelet and inhibit the response to AHGG, exerted no effect on the platelet response to H-CRP. Further, a functional receptor for thermally modified human or rabbit CRP was detected on rabbit platelets in the absence of a demonstrable Fc receptor for aggregated IgG. These data indicate that the platelet receptors for the modified forms of CRP and IgG are distinct. Thus, CRP seems to differentially regulate platelet function in the acute phase depending upon its physicochemical state and/or its association with other molecules.

OVERVIEW

In 1930, Tillett and Francis observed that sera obtained from patients during acute febrile illnesses have the ability to precipitate with an extract of the pneumococcus, designated Fraction C (C-substance) and later C-polysaccharide. Tillett, Goebel and Avery (1930) termed this serum material, "C-precipitin" and Ash (1933) showed that it occurred in non-febrile illnesses caused by both Gram-positive and Gram-negative bacteria. This material also was found in the sera of patients with rheumatic fever and other diseases of bacterial origin, and Abernethy and Francis (1933) observed that the C-substance could induce cutaneous reactions selectively in patients who had the C-precipitins in their sera. Abernethy and Avery (1941) presented evidence that the C-precipitin was a protein and demonstrated that calcium was required for it to react with the C-substance. They proposed that the C-reactive substance was distinct from antibody because it occurred only during the acute stages of infection, occurred in a variety of infections and non-infectious illnesses independent of the inciting agent, and was present in the albumin rather than in the globulin fractions during precipitation with ammonium sulfate.

MacLeod and Avery (1941a, b) extended these observations and purified the protein, demonstrating its immunologic specificity by both precipitation and complement fixation tests with antisera raised in rabbits to the purified material. Now termed C-reactive protein, or CRP, it can be detected and/or quantified by

precipitation with specific antisera, precipitation with the C-substance, or by calcium-dependent capsular swelling reactions with appropriate strains of pneumococcus (Lofstrom, 1944); of these, precipitation with specific antibody is most sensitive, rapid, and convenient. The widespread use of these tests extended the range of clinical conditions during which serum CRP was readily measurable, and revealed that a feature common to these conditions was the presence of reactions of acute inflammation or tissue destruction, particularly in inflammatory and malignant diseases (Anderson and McCarty, 1950; Shetlar et al., 1955). Thus, an elevated CRP level has served as a nonspecific indicator of the presence of these processes, rather than as an indicator of a specific group of diseases. As such, CRP has become known as the prototypic acute phase reactant.

The elevated levels of CRP detected in the circulation during the acute phase has justifiably raised the question of its role during inflammation and tissue destruction. The numerous functional similarities between CRP and immunoglobulin including the ability to initiate reactions of precipitation, agglutination, capsular swelling, opsinization and activation of complement (reviewed in Gewurz et al., 1982), suggests that CRP may serve as a non-immune effector in analogy to immune-mediated reactions. Such a role for CRP would be supportive of early participation in host defense mechanisms in the absence of immune protection. In contrast, CRP and immunoglobulin are distinguishable on the basis of nonspecific induction, antigenicity and lack of heterogeneity. Therefore, CRP may have additional biological functions whose purpose(s) are just now being appreciated; these may pertain to a role for CRP in the modulation of mechanisms involved in specific host resistance to infection, immune induction, tissue repair and homeostasis.

A single detailed discourse into all CRP biology is beyond the scope of this review. I have, however, collected our investigations into the effects of CRP upon platelet reactivity. The work of this laboratory and Department, which have as a major commitment the study of the acute phase response, has demonstrated that the interactions between CRP and the platelet are the most promising approach to the study of CRP-cellular interactions. This has become evident over the past few years as the effects of CRP upon platelet function have been the most consistent and sensitive indicator of CRP-cellular biologic function. It is possible that platelets represent the most important of the formed elements of the blood whose activity is in some manner regulated by CRP. Platelets are known to function in numerous capacities including maintenance of endothelial integrity, blood clotting, hemostasis and thrombosis, fibrinolysis, wound-healing and immune and inflammatory reactions. The functions attributed to platelets have in common the aegis of being a host response to injury or a defense mechanism. The modulatory effects that CRP exerts upon the platelet suggest that the acute phase in general may play a major role in regulating host responsiveness to insult and may, indeed, be a homeostatic response in the body economy. Moreover, since the internal events that precede the biologic expression of platelet function are in many ways similar to those of other cell types (e.g. lymphocytes, polymorphonuclear neutrophils, monocytes, basophils, and mast cells), knowledge of how CRP and other reactants modulate the response of the platelet to normally active stimulatory agents may yield information applicable to other cellular systems.

MATERIALS AND METHODS

(Fiedel et al. 1982a-d and Simpson et al. 1982)

Isolation of CRP

CRP was isolated from pleural or ascitic fluids obtained from patients undergoing diagnostic/therapeutic procedures. The fluids were centrifuged at 5000 g (15 min; 4°), further clarified by filtration through gauze and applied by gravity drip to a phosphocholine (PC)-Sepharose 4B affinity column (2.5 × 22 cm, Pharmacia Fine Chemicals, Piscataway, N.J.). The column was washed with 20 mM Tris-buffered saline containing 10 mM $CaCl_2$ (TBS^+; pH7.5) at 20 ml/hr for 36-48 hr after the A_{280} reached< 0.05; elution of CRP was achieved using isotonic Tris-citrate buffer, pH 7.2, at a flow of 20 ml/hr. Fractions containing CRP, as judged by immunoassay, were pooled and exchanged on a DE-52 (Whatman Ltd, Kent) column (1.5 × 20 cm) with elution achieved using a 0.1 - 0.4 M linear salt gradient (20 ml/hr); CRP-containing fractions were pooled and concentrated in an Amicon UF cell (PM-10 membrane) (Amicon Corp., Lexington, Mass.) and sieved through a 3 × 40 cm Sephacryl S-200 superfine column (Pharmacia; S-200) in Tris-buffered saline in the absence of $CaCl_2$ (TBS; pH 7.2). In a number of experiments, CRP preparations obtained from the PC affinity column were directly passaged over S-200. All final CRP preparations were dialyzed 1-3 days against 1000 vol of TBS before use. Final CRP concentration was determined by radial immunodiffusion (RID), Lowry and Bio-Rad (Richmond, Calif.) protein analyses and absorbance at 280 nm using an average extinction coefficient of 19.0 (g/dl). The purity of CRP was assessed by polyacrylamide gel electrophoresis (PAGE) in SDS and urea, and by a RID and double diffusion screen using a battery of monospecific antisera selected to detect components which we and others have observed to co-purify with CRP, or in which we have particular interest. At a minimum, this involved testing with antisera to IgG, IgM and IgA, serum amyloid P component, C3 and human serum; many preparations also were tested using antisera directed against albumin, ceruloplasmin, Clq, Clr and Cls, fibrinogen, fibrinopepitdes D and E, α and β-lipoproteins and orosomucoid. Reactions in RID were considered negative only after eight-fold application of highly concentrated CRP material such that, in most instances, 1-3 μg/ml non-CRP antigen could be detected in preparations containing 1.5 - 2.0 mg/ml CRP. Similarily, 50-100 υl (1-2 mg/ml) of CRP was subjected to SDS-PAGE to allow detection of trace amounts of non-CRP material. Final preparations of CRP were filter sterilized (Millipore Corp., Boston, Mass.) and stored at 4° in dosette vials. Marker molecules of known molecular weight which were used as standards in column chromatography and SDS-PAGE were obtained from Pharamacia Fine Chemicals.

Heat modification of CRP

CRP (500 μg/ml in TBS) was heat-aggregated (H-CRP) by incubation at 63° for 2.5 - 3.0 min and the aggregates collected by centrifugation (1000 g; 22°). The concentration of H-CRP was established by antigenic analyses of the CRP solutions before and following removal of the aggregates after heat-modification and by Lowry protein analysis of the washed aggregate, with virtually identical results. Between 47% and 53% of CRP was removed from solution during heat-modification.

Preparation of CRP-ligand complexes

Complexes were formed between CRP and two of its ligands, C-polysaccharide (CPS) and the synthetic polycation, poly-L-lysine (PLL; 15,000 mol. wt). These complexes were generally formed with CRP at a final concentration of 500 μg/ml in a total reaction volume of 200 μl. Complexes of CRP:CPS were formed at ratios of 10:1 to 2:1 CRP:CPS (w/w) during incubations at either 22° or 37° for 15-120 min, with an occasional further overnight incubation at 4°. All precipitate complexes were collected by centrifugation at 2000 g (22°; 10 min), the supernates removed and the pellet resuspended in 50 μl TBS. CRP:PLL complexes (25:1 to 10:1, w/w) were similarly prepared with the exception that incubation was carried out at 37° for 15-30 min. When individual complexes were pooled, the precipitate from a number of reaction tubes was transferred to a single tube and resuspended in a total of 50 μl TBS. CRP:CPS complexes, which form only in the presence of calcium, were prepared in TBS containing 1.2 mM $CaCl_2$; CRP:PLL complexes were prepared in TBS as their formation is markedly inhibited by the presence of calcium. Latex-aggregated CRP (Latex-AggCRP) was prepared by incubating 40 μl of a 1/10 dilution of washed latex beads (Bacto Latex 0.81, Difco, Detroit, Mich.; used as supplied) in 500 μl CRP (540 μg/ml) for 30 min at 37°. The beads were washed once and resuspended in 250 μl saline. Latex-aggregated IgG (Latex-AggIgG) was similarly prepared. In some instances it was necessary to adjust the number of latex beads or the stock concentration of CRP in order to maximize the platelet response. Calcium (1.2 mM) was present during the formation of Latex-AggCRP (or IgG). As native CRP does not interfere with the activation of platelets by Latex-AggCRP, the washing of these beads need not be routinely employed. Washing of Latex-AggIgG was required as native IgG interfered with the platelet activation stimulated by Latex-AggIgG.

Isolation of platelets and platelet activation

Washed human platelets were prepared from platelet-rich plasma (PRP) by sequential centrifugations and washing in Tris-EDTA buffer, pH 7.5, and resuspended at 3-5x 10^8/ml in a diluent consisting of three parts 0.5% glucose in normal saline and five parts 0.09 M Tris, 30 mM NaCl and 0.8 mM $CaCl_2$, adjusted to pH 7.5. Platelet aggregation was monitored in a Model 300 BD aggregometer (Payton Associates, Buffalo, N.Y.) by equilibrating TBS (300 μl) with 450 μl of PRP or the isolated platelet suspension for 1 min at 37° (1000 r.p.m.), adding the test agent, and monitoring aggregation responses for a minimum of 4 min. The simultaneous measurement of platelet aggregation and secretion was performed in a Model 400 Lumi-aggregometer (Chrono-Log Corp., Havertown, Pa.); the instrument utilizes the luminescent firefly luciferase system to detect secreted ATP with aggregation measured by turbidometric techniques. Reactions were performed at 37° in siliconized aggregation cuvettes (Chrono Log Corp.) with a stirring speed of 1200 r.p.m. The mixture consisted of 450 μl PRP or the isolated platelet preparation and 50 μl Chronolume reagent (luminescence assay mixture; Chrono-Log Corp.) prepared as directed; the platelet agonist (in TBS) was added and aggregation/secretion monitored on a dual channel recorder. In some experiments either CRP or H-CRP was included in the reaction mixture before challenge with the platelet stimulator.

Conversion of platelet [^{14}C]-arachidonic acid to [^{14}C]-TXB_2.

Blood was collected into 50 ml syringes, mixed with bisodium citrate-dextrose-

phosphate (9:1 v/v) and centrifuged at 200 g for 20 min to obtain PRP. The PRP was adjusted to 3 × 10^8/ml and incubated with TLC rechromatographed [^{14}C]-arachidonic acid (0.05 μCi/ml) at 37° for 60-90 min. The labeled platelets were then isolated by sequential centrifugations at 2000 g for 15 min and suspended in TBS at a concentration of 5 × 10^8/ml.

To assay the generation of [^{14}C]-TXB_2 (thromboxane B_2), the hydrated end-product of TXA_2 (thromboxane A_2), 1 ml of platelets was stirred (1000 r.p.m.) in the aggregometer, 10-100 μl of the agonist added and the reaction allowed to proceed for 5 min. Five microliters of [^{3}H]-TXB_2 (125 mCi/mmol) were added as an internal standard to determine the recovery of [^{14}C]-TXB_2 generated during the reaction. The reaction was terminated by addition of an aliquot of 1 M citric acid (10-20 μl) sufficient to reduce the pH to 3.5. The platelet mixtures were decanted into 10 ml of ethyl acetate (4°) in 13 × 125 mm acid washed glass culture tubes. The aqueous and organic layers were intermixed for 5 min using a paint shaker (Dayton Electric Co., Chicago, Ill.) and the mixtures centrifuged at 2000 g for 10 min (4°), resulting in upper-organic and lower aqueous phases. The extraction was repeated, organic layers pooled and evaporated under nitrogen at 32°, and dissolved in 1 ml of methanol.

For thin layer chromatography (TLC), the methanolic solution was evaporated to dryness and dissolved in 25-30 μl of methanol. The extracted lipids were applied to 250 micron silica gel G-60 plates and chromatographed at 22°. TLC plates were run in a first solvent of chloroform:acetic acid (90:1 v/v) to remove arachidonic acid and the hydroxyacids from the origin. The plates were then rotated 90° and chromatographed in a second solvent of diethylether:methanol:acetic acid (90:2:2 v/v) to separate prostaglandins E_2, $F_{2\alpha}$ and TXB_2. To assess the location of radioactive [^{14}C]-TXB_2, the thin-layer plates were scanned on a radiochromatograph scanner (Packard Model 7230); non-radioactive standards of prostaglandin E_2 and D_2 were chromatographed in the second solvent to determine the location of these prostaglandins (detected with iodine vapor). For a quantitative determination of TXB_2 generation, 1 × 1 cm areas were partitioned on the silica gel plates and those radioactive areas containing the appropriate lipids were scraped and eluted into scintillation fluid and counted. The total radioactivity of [^{14}C]-TXB_2 was corrected for background, percentage recovery of [^{3}H]-TXB_2, and the final results were expressed as the percentage of total platelet [^{14}C]-arachidonic acid converted to [^{14}C]-TXB_2. Total platelet [^{14}C]-arachidonic acid incorporation was determined by removing a 1 ml aliquot of the final platelet suspension, washing it in TBS and counting the pellet. The production of radioactive prostaglandins (PGE_2 and $PGF_{2\alpha}$) was qualitatively assessed.

Generation of thromboxane A_2 (bioassay).

Spirally cut strips of thoracic aorta, approximately 2 × 0.2 cm, were removed from anaesthetized male rabbits. Male rabbits were used because of their greater sensitivity to PGG_2/PGH_2 (prostaglandins G_2 or H_2) and TXA_2. Rabbits were anaesthetized by injection of 2-3 ml of a 50 mg/ml solution of Sodium Nembutal (Abbott Laboratories, N. Chicago, Ill.) into the ear vein using a 21 gauge butterfly. The aorta strips were suspended from an isotonic force transducer (Gould UC2) by surgical thread. The strips were continually superfused with Krebs-Bicarbonate buffer (37°) at a constant flow rate of 10 ml/min and aerated with 95% 0_2- 5% CO_2. Contractions were recorded with a polygraph, Model RPS 79C (Grass Instruments, Quincy, Mass.). A resting tension of 2 gm/cm was applied to strips

according to the calibration of the isotonic force transducer before the experiment. To increase the specificity of the tissue response, various pharmacological antagonists were included in the superfusate to inhibit tissue contractions to acetylcholine, serotonin, histamine, epinephrine, norepinephrine and arachidonic acid. These included 0.33 μM phenoxybenzamine, 0.25 μM mepyramine maleate, 0.9μM indomethacin and 1.4μM atropine (Sigma Chemical Co.; Sandoz Co., East Hanover, N.J.).

Platelet incubations were carried out in a final reaction volume of 0.5 ml in a Payton aggregation module. Precise kinetic analysis of the generation of TXA_2 activity for the platelet suspensions was done for each agonist. A dose response for platelets challenged with arachidonic acid was determined and a final concentration of 100 μM was used as a positive reaction control. Platelets, stirring in a cuvette, were preincubated for 1 min and then challenged with an activator, the contents of the cuvette drawn into a 1 ml tuberculin syringe and immediately administered into the injection portal superfusing the aortic strip. The time lag between removal from the reaction mixture and tissue perfusion was approximately 15 sec.

Pneumococcal C-polysaccharide and poly-L-lysine (PLL)

Pneumococcal C-polysaccharide (CPS) was the generous gift of Dr. Carolyn Mold-DuClos, Rush University, Chicago, Ill. CPS was prepared at 0.5 mg/ml in isotonic saline and stored at $-70°$. PLL (15,000 Daltons) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Radiochemicals

Radioactive materials were obtained as follows: [^{14}C]-5HT (serotonin), Amersham-Searle Inc., Arlington Heights, Ill.; [^{3}H]thromboxane B_2 (100-150 mCi/mmol), [^{14}C]-arachidonic acid (40-60 mCi/mmol) ([^{14}C]-AA) both from New England Nuclear, Boston, Mass. Aβ-thromboglobulin radioimmunoassay kit was obtained from Amersham-Searle, Inc. Nonradioactive prostaglandins E_2, D_2, $F_{2\alpha}$ and thromboxane B_2 (TXB_2) were the generous gift of Dr. J.E. Pike, Upjohn Pharmaceutical Co. Kalamazoo, Mich.

RESULTS

1. Description of the inhibitory effects of certain CRP preparations on platelet function.

As early as 1976 (Fiedel and Gewurz, 1976a, b) we had demonstrated that CRP could modulate certain platelet reactivities. CRP preparations were first found to inhibit the aggregation of washed platelets stimulated by both heat-modified human immunoglobulin (AHGG) and by thrombin. The inhibition was dose-dependent, not due to chelation of calcium or cytotoxicity, and characterized by reductions in the rate and maximal extent of aggregation and in the size of individual aggregates. Inhibition was not seen in the presence of known CRP ligands phosphorylcholine or C-polysaccharide, suggesting that it was mediated by CRP. Inhibition also was overcome by larger amounts of AHGG or thrombin, indicating an agonist-antagonist relationship dependent upon the relative concentrations of both the stimulating agent and CRP. The amounts of CRP required to inhibit platelet aggregation induced by thrombin (by 90%) was 10-fold greater than the amount required to inhibit AHGG. CRP preparations similarly inhibited

the generation of platelet factor 3 (PF3) activity by AHGG and thrombin and routinely reduced the release of β-glucuronidase following platelet challenge with thrombin, although extraordinarily high amounts of CRP were required to observe this latter effect. The inhibition of CRP was evident, though reduced, following preincubation of the platelets with CRP and subsequent washing of the platelets; CRP could not be found to interact with AHGG or thrombin as determined by sucrose density sedimentation profiles of ^{125}I-CRP alone or in combination with each agent and CRP failed to affect the thrombin-fibrinogen clotting time. Further study of the effects of CRP upon thrombin-induced platelet activation revealed that the inhibition of platelet aggregation coincided with the decreased release of ADP, serotonin (5HT) and a non-prostanoate degradative product of PGH_2, malondialdehyde. This inhibition occurred in the absence of an effect upon basal levels of platelet cAMP.

CRP was subsequently found to inhibit platelet response in citrated plasma stimulated with ADP, epinephrine or collagen (ASC). The inhibitory effects of CRP following platelet challenge with ADP or epinephrine were associated solely with second wave aggregation and concomittant with decreased secretion of 5HT (Fig. 1) and the generation of second-wave associated PF3 activity. CRP preparations also inhibited collagen-stimulated platelet aggregation in citrated plasma (data not shown) in the absence of any observable effect upon collagen-platelet adhesion (Table 1). CRP did not alter platelet shape change following collagen addition and greater amounts of CRP were required to effectively inhibit collagen induced aggregation than were required to effectively inhibit second-wave ADP- and epinephrine stimulated platelet responses.

CRP was found not to inhibit indomethacin-insensitive platelet responses stimulated with the calcium ionophore A23187 (Fig. 2) nor the indomethacin and 5,8,11, 14 eicosatetraynoic acid (ETYA)-insensitive secretory response initiated by semi-purified platelet activating factor. By contrast, CRP readily inhibited platelet aggregation stimulated with arachidonic acid (Fig. 3) (Fiedel, Simpson and Gewurz, 1977). However, there existed an inconsistancy in the biologic effects of CRP upon the platelet. Although the majority of CRP preparations (87%) inhibited platelet activation, this effect was often short-lived being lost upon storage; such loss was associated with the generation of insoluble material in the CRP preparations, previously suspected to be aggregates of CRP. A putative role for an accessory molecule in these reactions now has been obtained.

2. Evidence for a low molecular weight factor in CRP-associated inhibition of platelet function.

CRP isolated from ascites or pleural fluids by PC (phosphocholine)-affinity column chromatography was passaged over Sephacryl S-200 and selected fractions were pooled and tested for the ability to inhibit platelet aggregation stimulated with ADP or acid soluble collagen (ASC) in both the absence and presence of C-polysaccharide (CPS), a known ligand of CRP (Fig. 4). Platelet inhibitory activity generally was observed in two eluant zones, the first involving a factor migrating as a species of 115,000 molecular weight (mol. wt.) coincident with CRP antigenicity (main peak) and the second involving a low molecular weight factor (LMF) migrating as an apparent species of 8300-12,500 mol. wt. Only the inhibition observed with the former was abrogated in the presence of CPS sug-

gesting that the platelet inhibitory activities of CRP and LMF were distinct. However, rechromatography of the CRP main peak over Sephacryl S-200 yielded CRP devoid of platelet inhibitory activity as well as a separated low molecular weight factor, reflective of a close relationship between the two molecules.

In order to more critically assess the presence of LMF, portions of the PC-affinity eluates were passaged over a mini-column of Sephacryl S-200 and CRP antigenicity and platelet aggregation inhibitory capacity were evaluated for each fraction (Fig. 5) (Fiedel et al., 1982a). A single large broad protein peak and a second smaller area of absorbance cooresponding to the molecular weight of the ribonuclease A marker were observed. CRP antigencity was present throughout the first peak and anomalously present in the second peak, corresponding to a molecular weight slightly less than 12,700. When this low molecular weight region containing LMF activity was analyzed by SDS-PAGE (following concentration), both an apparent 23,000 dalton CRP subunit component and a larger »240,000 mol. wt. component were detected. When LMF prepared in this manner was concentrated by Amicon ultrafiltration and then rechromatographed over Sephacryl S-200, a peak eluting at 115,000 mol. wt. (antigenic identity to CRP: 23,000 mol. wt. in SDS-PAGE) as well as a large molecular weight peak free of CRP antigenicity eluting at the column Vo (»200,000) were observed; little low molecular weight material was visualized. This suggested that under appropriate conditions (concentration?) the low molecular weight component might assemble into aggregates. The high molecular weight component did not inhibit platelet activation.

Since the PC-affinity procedure followed by a single gel sieve chromatography step generally did not adequately isolate CRP free of LMF, we attempted to ascertain whether an ion exchange chromatography procedure intermediate between the PC-affinity isolation and molecular sieve chromatography steps better served the purification of CRP. CRP which eluted from the PC-affinity column was exchanged on DE-52 before passage over Sephacryl S-200 (Fig. 6). Chromatography of the PC-affinity eluate over DE-52 yielded material eluting at 0.24-0.27 RSC (relative salt concentration) which migrated as a single 115,000 mol. wt. peak over Sephacryl S-200, gave a 23,000 dalton component in SDS-PAGE and had an A_{280}:A_{260} ratio of 1.9-2.1; these data are representative of 34 of 40 preparations. In 6 of 40 preparations (Fig. 7), the PC-affinity eluate resulted in an eluate from the DE-52 anion exchange resin at 0.17 RSC (peak 1) as well as at 0.27 RSC. The peak 1 material contained CRP antigenicity, had an A_{280}:A_{260} ratio of $\sim$1.1 and after concentration gave both 23,000 and »240,000 mol. wt. components in SDS-PAGE. Peak 1 was resolved into two major peaks by passage over Sephacryl S-200, one containing and the other lacking CRP antigenicity; the latter peak had an extremely low A_{280}:A_{260} ratio (0.4), chromatographed on Sephacryl S-200 with an elution volume identical to that of the low molecular weight factor, gave a molecular weight of »240,000 in SDS-PAGE (after concentration) and precipitated from solution within 3-6 h of isolation. The material which eluted from the ion exchange column at 0.24-0.27 RSC (peak 2), when resolved over Sephacryl S-200, chromatographed identical to the 0.24-0.27 RSC material described previously and gave a single 23,000 mol. wt. component in SDS-PAGE. These data pointed to the appropriateness of an intermediate ion exchange chromatography step for the purification of each CRP-LMF and of CRP free of LMF.

The materials eluted from these columns were tested for their ability to modulate platelet activation stimulated by ADP, ASC, and arachidonate in both

the absence and presence of C-polysaccharide. As expected, CRP with A_{280}:A_{260} ratios of 1.9-2.1 did not inhibit platelet activation in amounts of 200 μg/ml. However, in the presence of LMF (whether isolated as CRP-LMF [A_{280}:A_{260} ratio of $\sim$1.1] or obtained by recombination of the separated components), inhibition of platelet activation stimulated by ADP, ASC and arachidonate was observed in a manner consistent with previous reports (Fiedel and Gewurz, 1976a, b; Fiedel, Simpson and Gewurz, 1977). Morever, this inhibition was not evident in the presence of the CRP ligand, CPS. These data imply that association with LMF was responsible for the platelet inhibitory activity attributed to native CRP, and that this association accounts for the sensitivity of this inhibition to the presence of the CRP ligand, CPS.

In summary, this material (LMF) often co-elutes (co-purifies) with CRP isolated from pleural or ascites fluids during CPS or PC affinity column chromatography, can be separated from CRP by ion exchange on DE-52 followed by molecular sieve chromatography over Sephacryl S-200; passage of these post-affinity eluates directly over S-200 bypassing the ion exchange step generally does not provide for adequate separation. Though not yet identified as to its molecular species, this factor has a nominal mol. wt. of 8500-12,000 as judged by column chromatography on Sephacryl S-200 and an A_{280}:A_{260} ratio of $\sim$0.4. Minimal concentration of LMF by Amicon ultrafiltration before rechromatography over S-200 results in the appearance of high molecular weight material (»200,000). This high molecular weight material does not possess the platelet inhibitory activity; thus, its relationship to LMF awaits further clarification. It is not yet clear whether LMF is comprised of protein, nucleic acid and/or nucleoprotein lipid and/or (apo-) lipoprotein; its ultraviolet absorption fingerprint with peak absorbance at $\sim$254 nm (Fig. 8) suggests that if LMF is a protein it is either lacking or relatively poor in tyrosine and tryptophan residues, or that such residues participate little in its absorption spectra.

LMF is not generally detectable by immunological analyses against a wide variety of antisera, although the association of LMF with CRP reduces the overall negative charge of the CRP molecule and isolated CRP-LMF exhibits a different antigenic array than purified CRP as judged by Ouchterlony and Mancini analyses. On occasion, we observe separated LMF to react with anti-sera against native CRP; the meaning(s) of this remains unclear. Collectively, these data supported our contention that CRP could serve to down-modulate platelet reactivity during inflammation and tissue destructive conditions, but that this modulatory capacity requires CRP to be associated with LMF.

3. *Definition of the platelet modulatory capacity of purified CRP when in complexed form.*

CRP shares many functional similarities with immunoglobulin including the ability to initiate reactions of complement consumption and phagocytosis and the capacity to bind with mononuclear peripheral blood cells *in vitro*. These reactivities each require CRP to bind with one of its multivalent ligands or to be heat-modified or chemically aggregated (modified) (reviewed in Gewurz et al., 1982). We questioned whether CRP, once separated from LMF, could be re-complexed (or altered) by physical means or by association with ligand to bring about a biological effect upon the platelet. The functional similarities of CRP and IgG suggested that such might be the case, and further, that this association might stimulate the platelet unlike CRP (LMF). Thermally aggregated CRP (H-CRP), but not

unmodified CRP, induced reactions of aggregation and constituent release from isolated platelets; maximum responses occurred with 40-80 μg/ml H-CRP and were similar to responses mediated by thermally aggregated human IgG (AHGG) (Figs. 9, 10) (Fiedel, Simpson and Gewurz, 1982b). Platelet aggregation was monophasic in nature and activation was sensitive to the presence of EDTA and dibucaine, required metabolic energy and was inhibited by increased levels of cAMP.

H-CRP participated with other platelet activators to synergistically bring about a platelet response *in vitro*. This is conceptually important since it has been hypothesized that *in vivo* platelet reactivity rarely is promulgated by any single activator but rather reflects the interactive involvement of multiple activators. AHGG also exhibited the capacity to operate synergistically with other platelet stimulators but, compared with H-CRP, was much less effective and required a greater basal level of platelet stimulation in order to be effective (Fig. 11).

Complexes formed between CRP and one major ligand class, the polycation (e.g. PLL and/or protamine), like H-CRP, also activated the platelet in an isolated buffer system (Fig. 12 and Table 2) (Fiedel, Simpson and Gewurz, 1982b, c). Since platelets are activated by various polycations (reviewed in Fiedel et al., 1976b), it is possible that polycations cross-linked by CRP might present a more positively charged particulate surface to the platelet to induce activation than does the soluble polycation alone. The inability to generate complexes between CRP and another ligand, CPS, that activate and/or interfere with activation of the platelet by H-CRP or CRP-PLL complexes may be the result of the absence of appropriately presented positive charges on CPS. Alternatively, the highly negative nature of CPS may itself render the complex inactive. However, the ability of H-CRP alone to activate the platelet and data which shows that CRP, like IgG, activates platelets when absorbed onto latex beads (see below), illustrates that CRP has an intrinsic ability to be cross-linked or altered in a manner suitable for platelet activation, and suggests that the polycation in CRP-PLL complexes may, in part, serve to appropriately cross-link CRP. Moreover, since CRP shows a distinctly different circular dichroism spectrum dependent upon whether calcium is present or absent suggesting the existence of two (or more?) allosteric forms of CRP, it is possible that the cross-linking of CRP molecules by heat, absorption onto latex beads or with an appropriate polycation (performed in the absence of calcium) might permit formation of a CRP aggregate which is in an allosterically favored form to activate the platelet. Those CRP aggregates formed in the presence of calcium or reacting with the phosphocholine (PC) moiety (e.g., with CPS) may not be in an allosterically suitable form. It is thus of interest to note that in the presence of calcium, CRP does not suitably heat aggregate nor will it form aggregates with polycation to form a complex capable of activating platelets. We synthesized bifunctional PC-substituted cationic homopolymers (e.g., PC-PLL) in order to test whether CRP complexes formed in the presence of calcium can activate the platelet given a sufficient distribution of positive charges; they clearly can (Fig. 13) (Fiedel, Simpson and Gewurz, 1982c).

Immunoglobulin G, aggregated by absorption to latex or polystyrene particles, has been found capable of initiating reactions of aggregation and secretion from isolated human platelets. We therefore determined whether CRP absorbed to latex beads, like H-CRP and CRP:PLL (or protamine), would initiate platelet reactivities. Not unlike IgG, CRP-coated latex particles activated the platelet to

reactions of aggregation and the secretion of dense body constituents (Fig. 14). Dilution experiments revealed that a minimum of 20 μg/ml CRP (coated on latex) was required to activate the platelet with maximal aggregation occurring at 40 μg/ml latex-aggregated CRP. Unlike H-CRP and AHGG (in which H-CRP was a 10-20 fold more effective platelet activator on a weight basis than AHGG), latex aggregated CRP and IgG were of approximately equal ability in the induction of a platelet response.

Continued studies of the interaction between CRP and the platelet now reflect that platelet activation stimulated by an aggregated CRP is sensitive to nonsteroidal anti-inflammatory drugs as well as the ADP-removing enzyme system creatine phosphate/creatine phosphokinase. Thin-layer chromatographic analysis of prostanoate endproducts following platelet activation with H-CRP revealed the formation of thromboxane B_2, the hydrated endproduct of thromboxane A_2, an important endogenous platelet activator and contractor of vascular tissue; bioassay on rabbit aorta strips of supernatants obtained from platelets undergoing challenge with H-CRP supported the TLC analysis (Fig. 15) (Simpson et al., 1982).

If one defines the relative potency of platelet activators into a hierarchy (Holmsen, 1977) dependent on their ability to stimulate the platelet responses of shape change, aggregation, endoperoxide and thromboxane production, dense granule and alpha granule secretion, then H-CRP has been previously shown to stimulate four of five basic responses. We have now determined that H-CRP stimulates the release of alpha granule secretion (Fig. 16) (Simpson et al., 1982), which is consistent with a platelet stimulatory potency equivalent to thrombin and collagen and superior to that of ADP or epinephrine (Holmsen, 1977). These data imply an agonist role for this acute phase reactant in platelet physiology and suggest that the interaction of modified CRP with the platelet at sites of vascular damage could have pathological significance.

4. Elucidation of the binding reaction between CRP and the platelet.

As stated before, CRP and immunoglobulin share many functional similarities including reactions of agglutination, opsonization, activation of complement and complement-dependent hemolysis and opsonic mechanisms (reviewed in Gewurz et al., 1982). Further, we have demonstrated that CRP as well as IgG, in their heat-aggregated or latex absorbed forms, can activate human platelets. This litany of overlaps between the ligand-binding and effector properties of the respective molecules led us to consider whether aggregated CRP might in fact utilize the IgG Fc receptor to initiate platelet activation.

We first approached this question by evaluating whether the expression of the platelet receptor for H-CRP and AHGG could be functionally separated; and established the comparative enzymatic sensitivities of the respective platelet receptors (CHY = chymotrypsin; PLC = phospholipase C; PLS = plasmin). Both the H-CRP and AHGG platelet receptors exhibited increased expression following treatment of platelet with CHY, but not following similar treatment with PLS or PLC (Fig. 16) (Fiedel et al., 1982d). The expression of platelet receptors for collagen (ASC) exhibited a different sensitivity profile, as do the platelet receptors for thrombin (Tam et al., 1980). These data illustrated a categorical similarity between receptors for H-CRP and AHGG as compared to receptors for ASC or thrombin; moreover, as CHY treatment of platelets releases much of the major glycoproteins from the platelet surface (Pfueller et al., 1977), it seems possible

that these glycoproteins mask platelet reactivity to both H-CRP or AHGG. However, differences in the sensitivity of the platelet response to H-CRP or AHGG in the presence of column fractions containing various plasma components revealed a distinction between these receptors, as did evidence that monomeric IgG substantially interfered with the platelet response to AHGG but not to H-CRP. Our recent descriptions of an H-CRP receptor on the surface membrane of rabbit platelets whose expression is also CHY sensitive and that is presently characterized by its ability to initiate both platelet aggregation and the formation of metabolic products from platelet arachidonate in the absence of demonstrable IgG Fc receptor activity (Fiedel et al., 1982d), further indicates that the platelet receptors for aggregated CRP and IgG are distinct.

CONCLUDING REMARKS

Data spanning the last eight years concerning the effects of CRP upon platelet function have been presented herein. In our opinion, CRP is a multi-functional molecule with respect to its ability to alter platelet behavior, being an agonist (or synergist) as well as an antagonist dependent upon is physical state and/or association with secondary molecules. There are clearly areas in which intensive research must be directed in order to ascertain the *in vivo* physiological significance of the CRP-platelet interaction: we must better define LMF and its distribution in the various disease states and evaluate its associative reaction with CRP; we must search *in vivo* and find complexed or physically altered forms of CRP with biologic potential directed towards the platelet, and we must describe them biochemically; we must also describe the platelet-CRP interaction at the membrane, fully understand its stimulus-response coupling, and map the platelet surface for CRP receptors; and lastly, as there is little reason to suspect that other acute phase reactants do not inter-play with one another to produce biologic phenomena, we must better understand these complex interactive relationships in the context of not only platelet function but, more generally, in terms of multiple biologic response modifiers during inflammatory and tissue destructive states. The future remains challenging.

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Table 1. Effect of CRP on collagen-platelet adhesion[a]

Expt. No.	CRP	Marker	% Mean Adherence ± S.D.		p
			Buffer	CRP	
	μg/ml				
1	45	^{14}C-5HT	8.1 ± 3.4	8.1 ± 3.1	> 0.2
2	45	^{51}Cr	11.9 ± 4.2	10.3 ± 3.4	> 0.2
3	100	^{51}Cr	8.3 ± 1.4	8.3 ± 1.3	> 0.2

[a] Appropriately labeled platelets (10^9) were incubated for 15 min with varying concentrations of CRP in 1.0 ml and added to collagen-coated tubes; these were rotated 15 rpm for 15 to 20 min at room temperature, decanted, and washed four times with modified Tyrodes solution. The bound ^{51}Cr was counted directly in a well-type gamma counter, while aliquots of solubilized platelets were assayed for ^{14}C-5HT by liquid scintillation. The results represent the percentage of counts bound compared to the total radioactivity of the platelet suspensions and are presented as the percent mean adherence of eight replicate samples ± one standard deviation (S.D.); p values were determined by the paired t-statistic.

Table 2. Aggregation of platelets by CRP:PLL and H-CRP complexes or by PLL

Reactant	Unseparated complex (%)	Supernate alone (%)	Precipitate alone (%)	PLL alone (%)
CRP:PLL (w/w)				
100:1	6	< 5	< 5	< 5
50:1	28	< 5	32	< 5
25:1	88	< 5	83	< 5
10:1	75	< 5	68	< 5
H-CRP (50 g/ml)	75	< 5	79	—

* CRP:PLL complexes were formed by addition of 20 μl of varying amounts of PLL to 1.0 ml of CRP (500 μg/ml) in TBS followed by incubation of 37°/15 min. PLL controls were prepared by adding equivalent amounts of PLL to 1.0 ml saline and represents the maximum PLL concentrations used to prepare the CRP:PLL complexes (1-10 μg/assay). H-CRP was prepared as described previously. These CRP aggregates were either used unseparated or the precipitates were collected by centrifugation (22°; 10 min) and resuspended in 1.02 ml (CRP:PLL) or 1.0 ml (H-CRP) saline, respectively. Two hundred microlitres of each CRP:PLL or PLL control preparation was then tested for the ability to aggregate platelets.

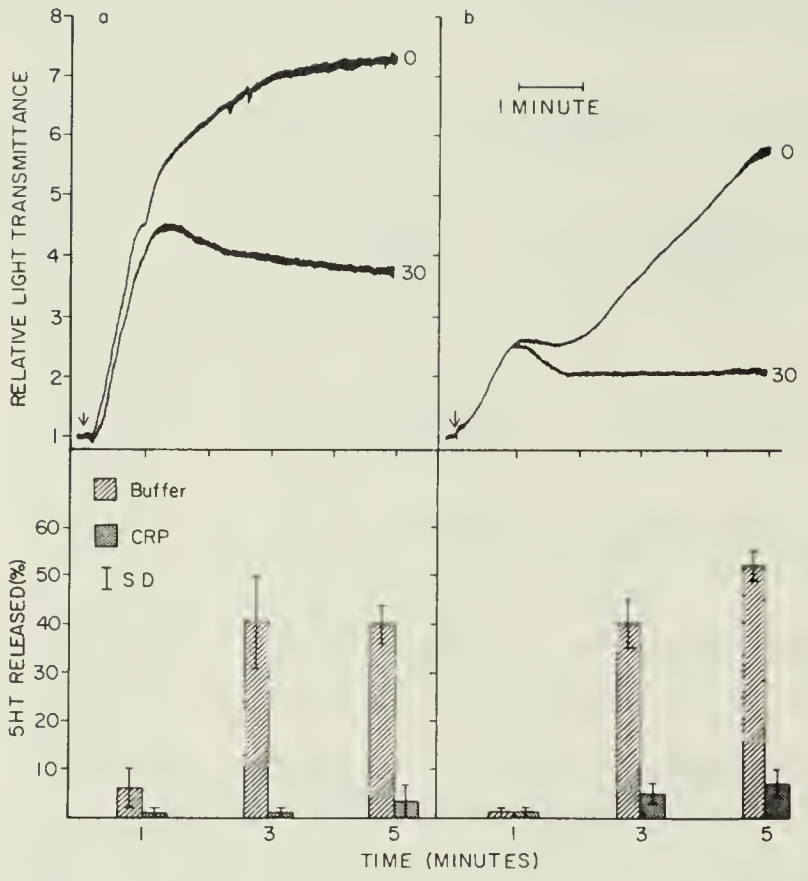

Fig. 1. Effects of certain CRP preparations (30 μg/ml) on the aggregation of and release of 5HT from platelets in PRP in response to: a) 10^{-5}M ADP and b) 10^{-5}M epinephrine.

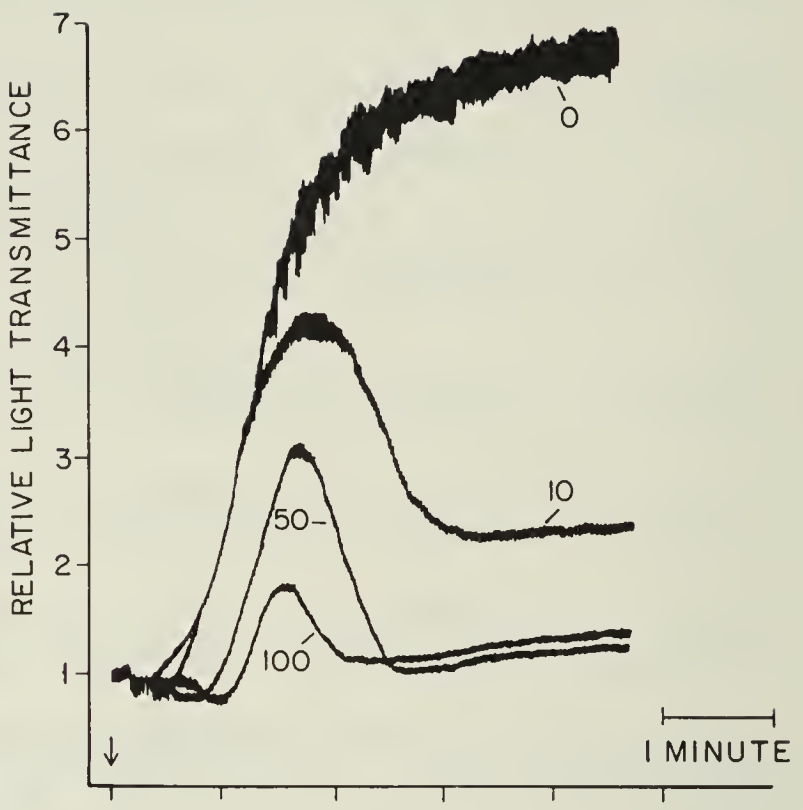

Fig. 2. Effects of certain CRP preparations on the aggregation of and release of 5HT from platelets in PRP in response to A23187: a) no CRP and b) 150 μg/ml CRP. The concentrations of A23187 are expressed as μM final concentration, and the percent release of 5HT is shown in parentheses.

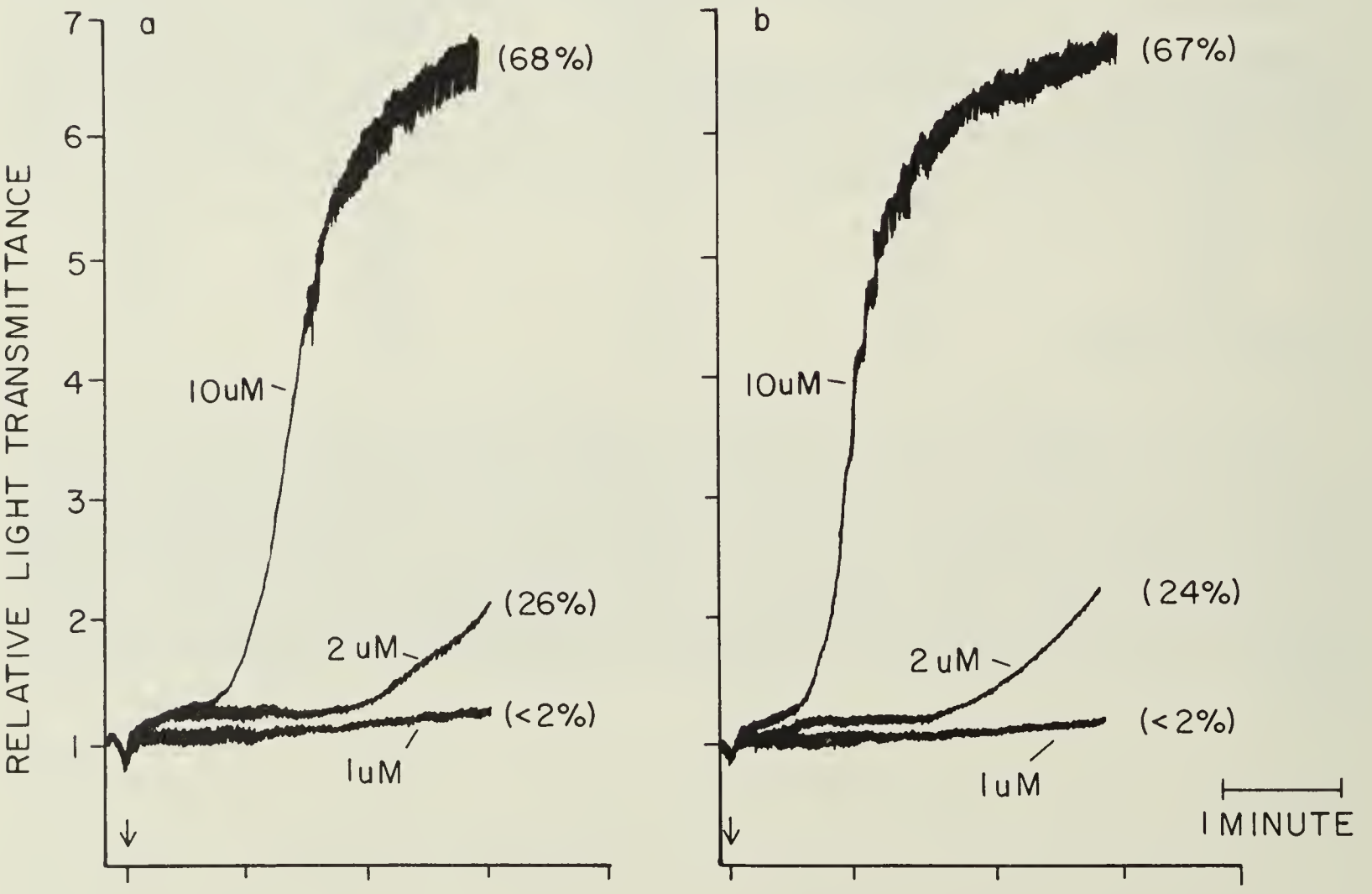

Fig. 3. Effect of certain CRP preparations on the aggregation of platelets in PRP in response to 0.5 mM arachidonic acid. The concentrations of CRP are expressed as μg/ml.

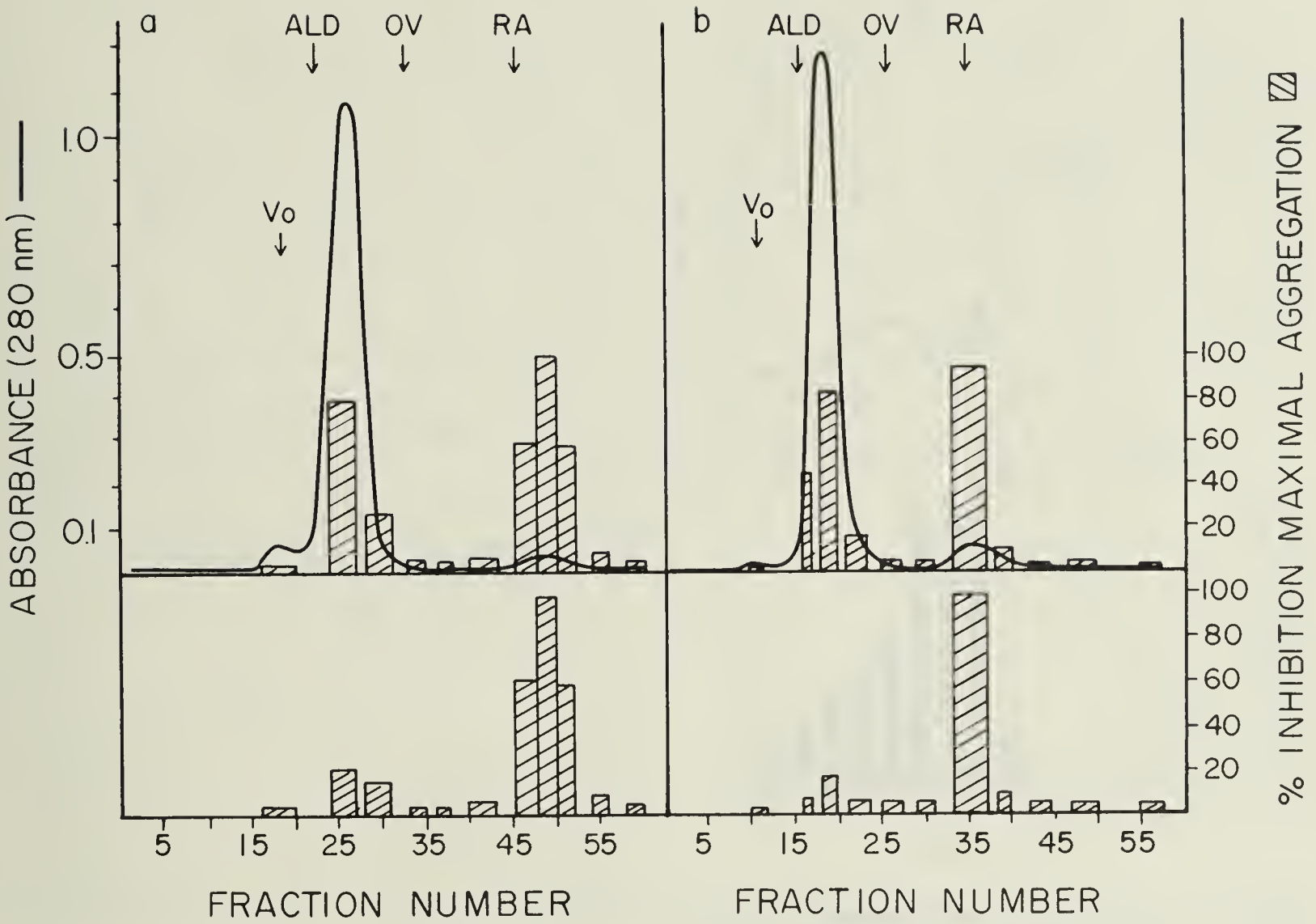

Fig. 4. Passage of CRP prepared by PC-affinity chromatography through Sephacryl S-200 and the inhibitory action of the various pooled fractions on platelet aggregation stimulated by ADP (a) or acid soluble collagen (b). Upper panels reflect the inhibition in the absence and lower panels in the presence of C-polysaccharide (CPS). CPS was added to CRP-containing fractions to obtain a constant ratio (10:1 by weight) of CRP:CPS; in fractions devoid of CRP antigen, CPS was added at a variety of concentrations, all without effect. The column was calibrated with blue dextran 2000, aldolase (ALD), ovalbumin (OV) and ribonuclease A (RA). Absorbance 280 nm (-); percentage inhibition maximal aggregation (▨).

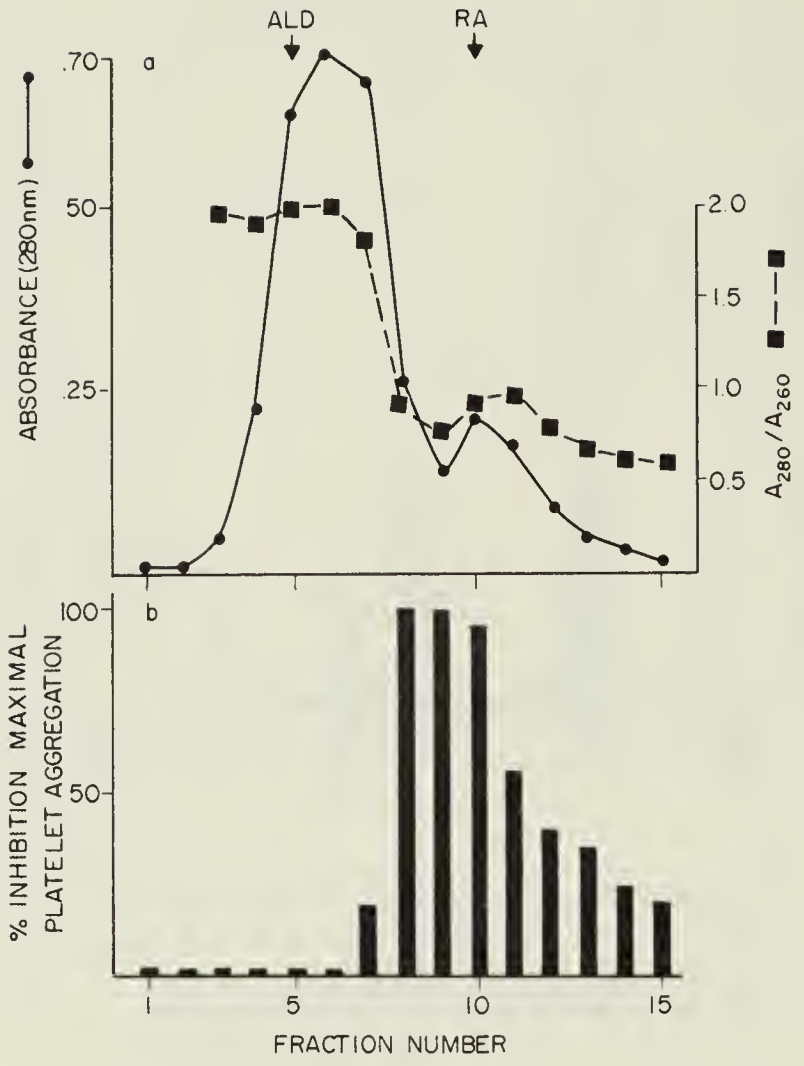

Fig. 5. Chromatographic separation of the PC-affinity eluate over Sephacryl S-200 resin (a) and inhibition of platelet aggregation by the various fractions (b). The A_{280}:A_{260} ratios are shown (■). ADP was used as the platelet stimulator at a concentration yielding 50% maximal irreversible aggregation; similar data was obtained with collagen as the platelet activator. The column was calibrated with ALD and RA. Absorbance: 280 nm (—).

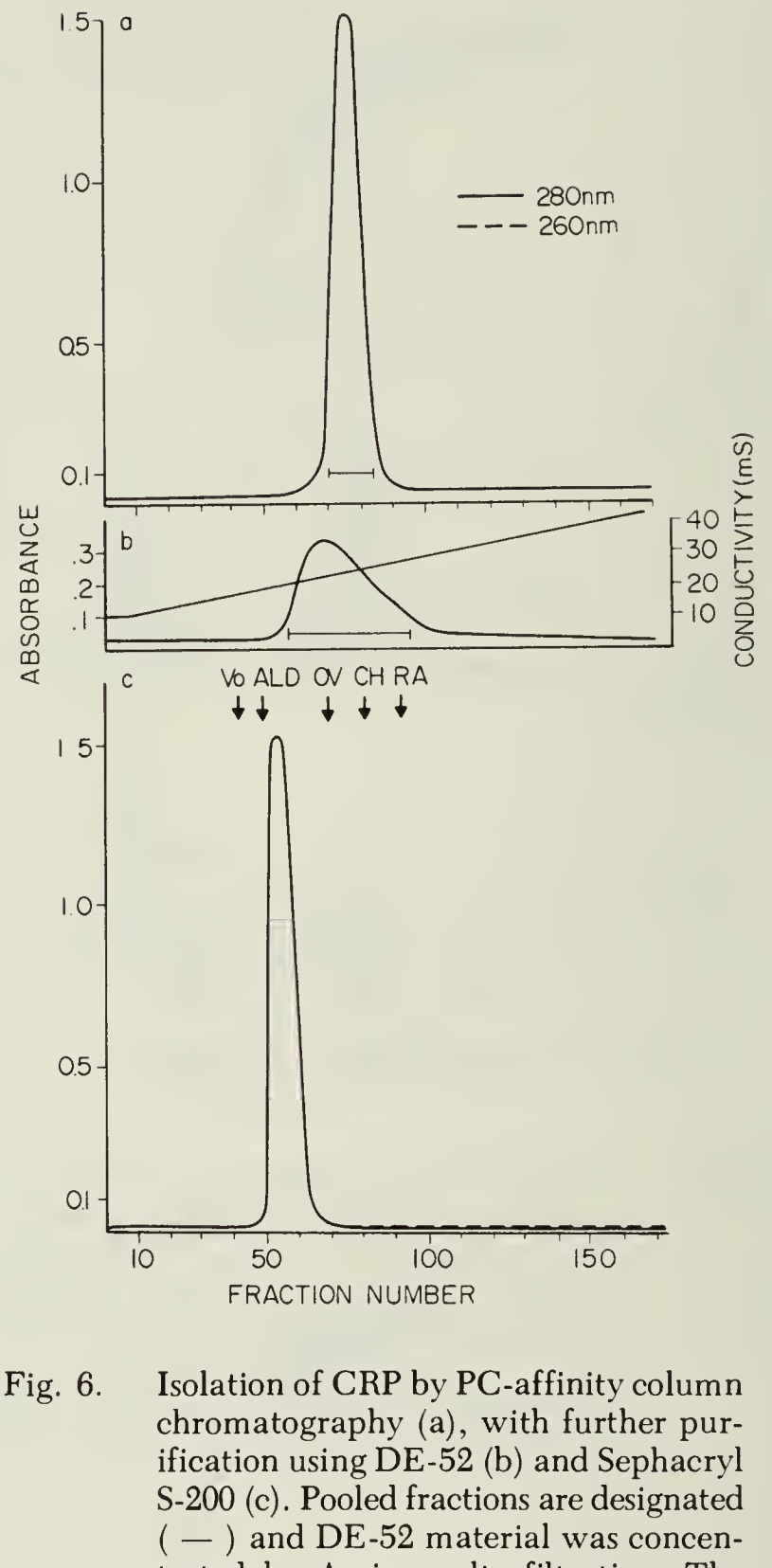

Fig. 6. Isolation of CRP by PC-affinity column chromatography (a), with further purification using DE-52 (b) and Sephacryl S-200 (c). Pooled fractions are designated (—) and DE-52 material was concentrated by Amicon ultrafiltration. The gel sieve column was calibrated with blue dextran 2000, ALD, OV, chymotrypsinogen (CH) and RA. Absorbance: (—) 280 nm; (---) 260 nm.

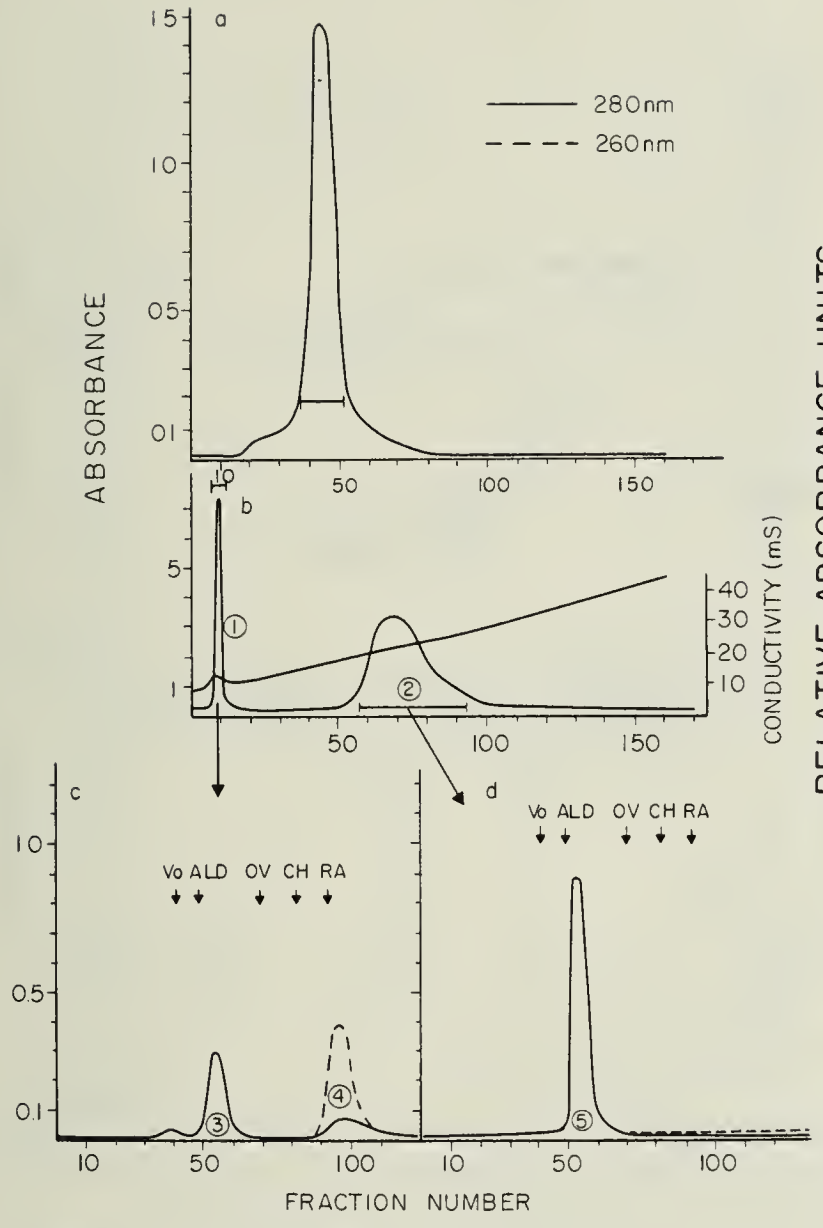

Fig. 7. Isolation of CRP by PC-affinity column chromatography (a), with further purifications using DE-52 (b) and Sephacryl S-200 (c,d). Pooled fractions are designated (—) and all DE-52 material was concentrated by Amicon ultrafiltration before gel sieve chromatography; the gel sieve column was calibrated as in Fig. 4. Absorption: 280 nm (—); 260 nm (---).

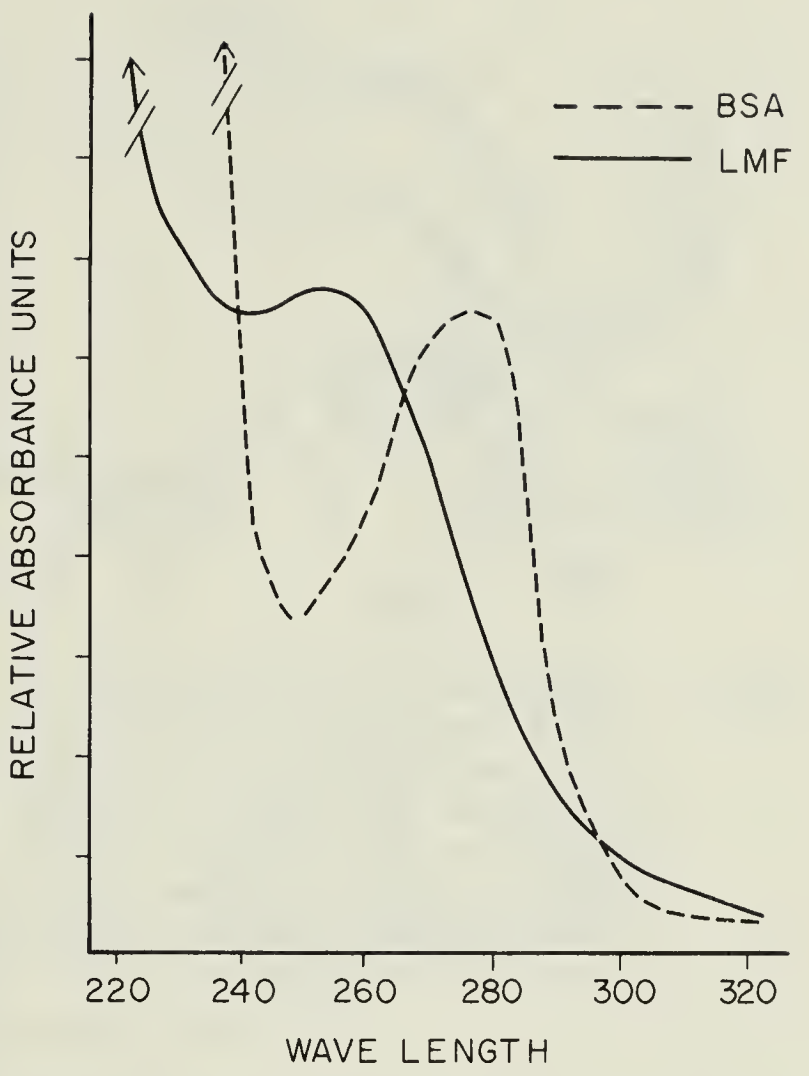

Fig. 8. Ultraviolet absorption spectra of the low molecular weight factor (—) and a reference protein, bovine serum albumin (---).

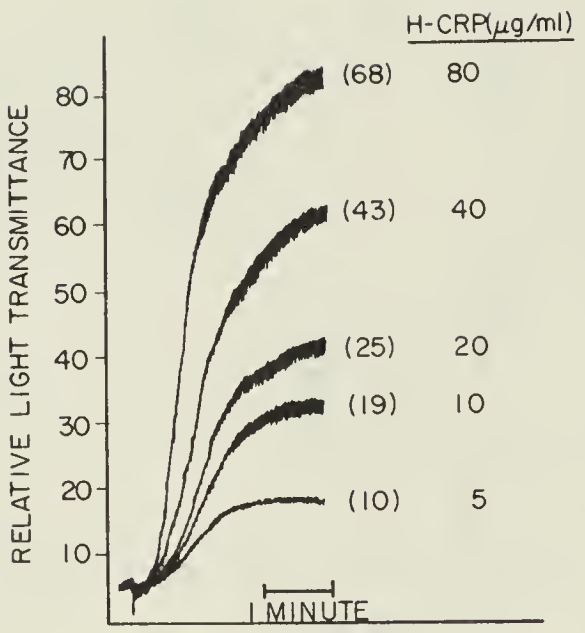

Fig. 9. Aggregation of human platelets following addition of various concentrations of heat-modified CRP (H-CRP) in buffered saline. The aggregation response was monophasic and similar to that observed with heat-modified human immunoglobulin G. The percent secretion of 5HT is shown in ( ).

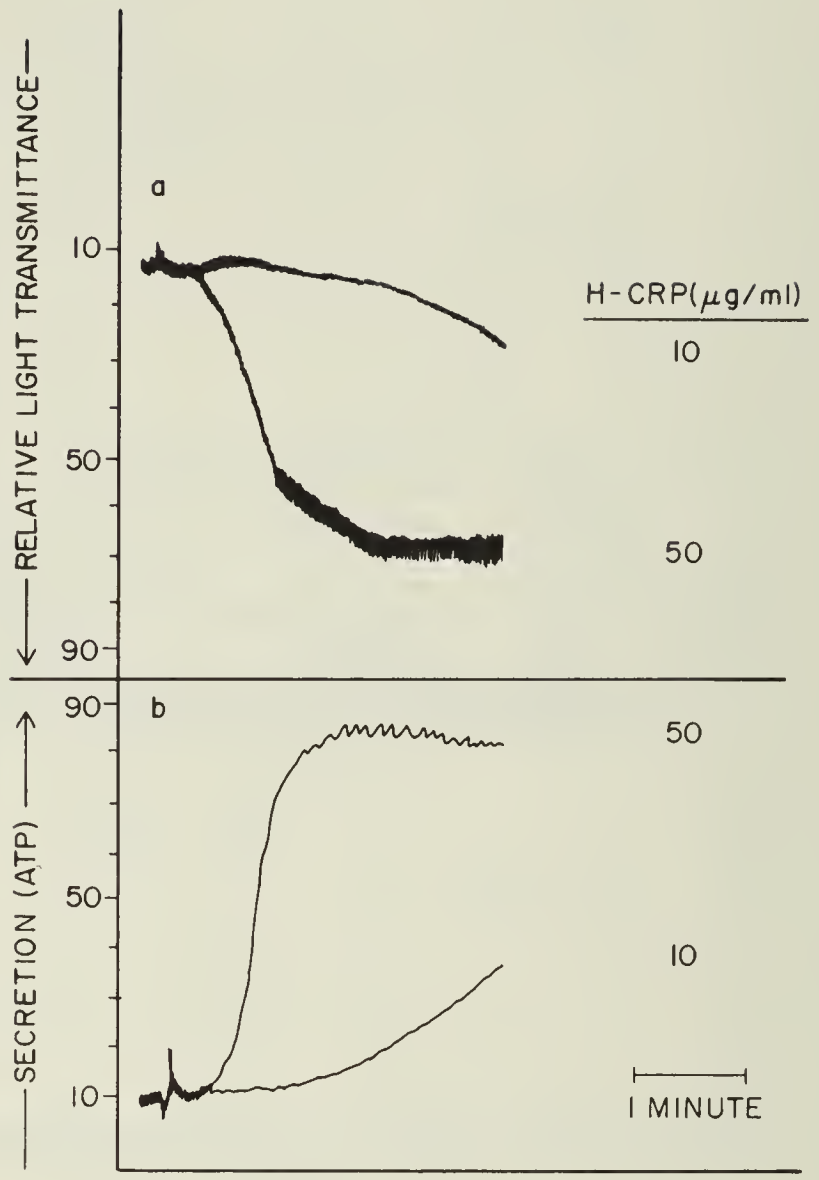

Fig. 10. Simultaneous comparison of platelet aggregation (a) and relative secretion of ATP (b) stimulated by heat-modified CRP (H-CRP) in buffered saline.

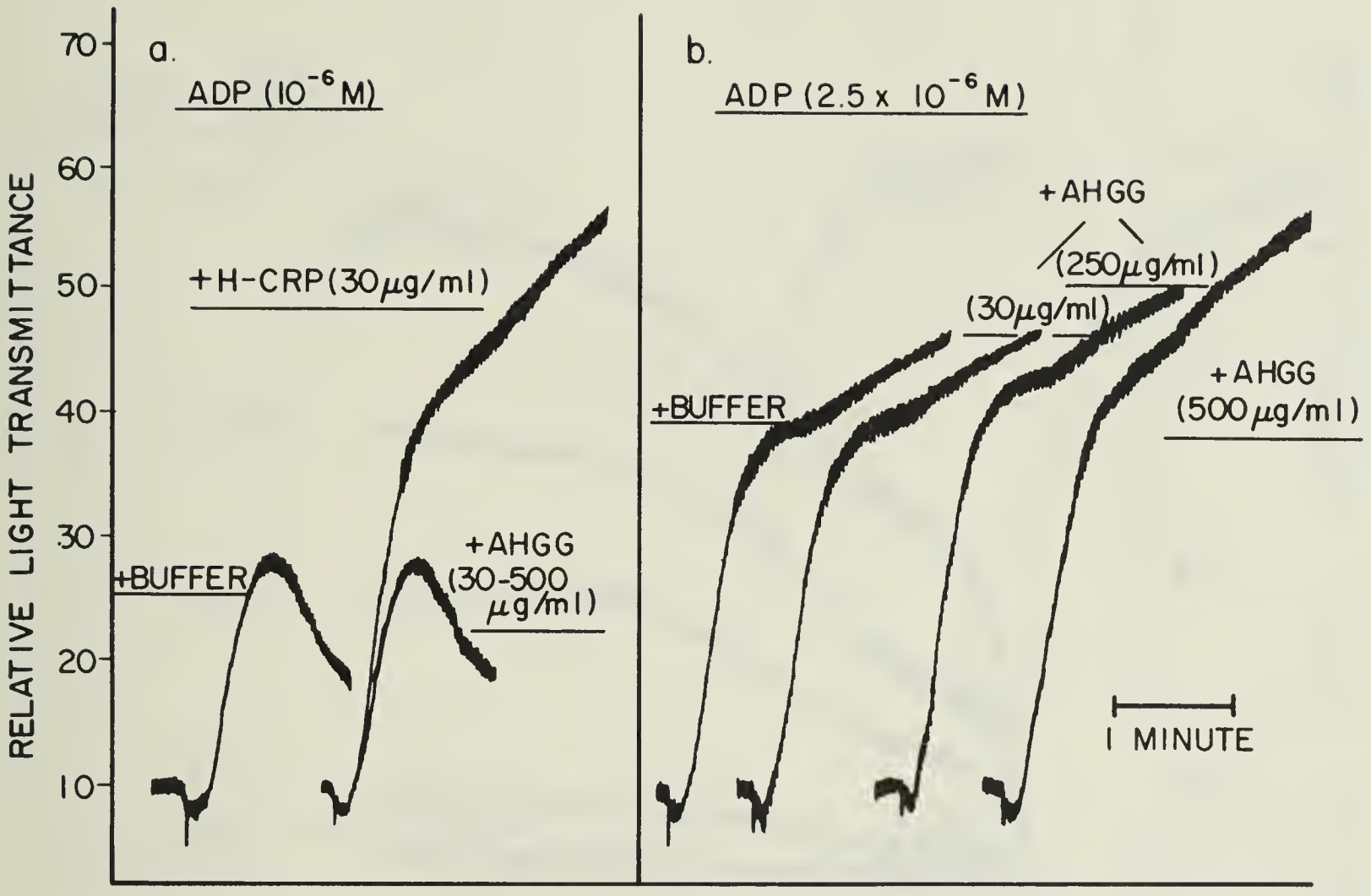

Fig. 11. Comparison of the ability of heat-modified CRP (H-CRP) or human immunoglobulin G (AHGG) to act synergistically with ADP in platelet-rich plasma. In (a) ADP was used at 10^{-6}M and gave a minimal level of activation while in (b), ADP was used at 2.5×10^{-6}M and yielded a more substantial level of platelet aggregation. H-CRP and AHGG were used at the indicated concentrations. H-CRP was, on a weight basis, at least tenfold more effective than was AHGG and required a lesser degree of basal platelet stimulation.

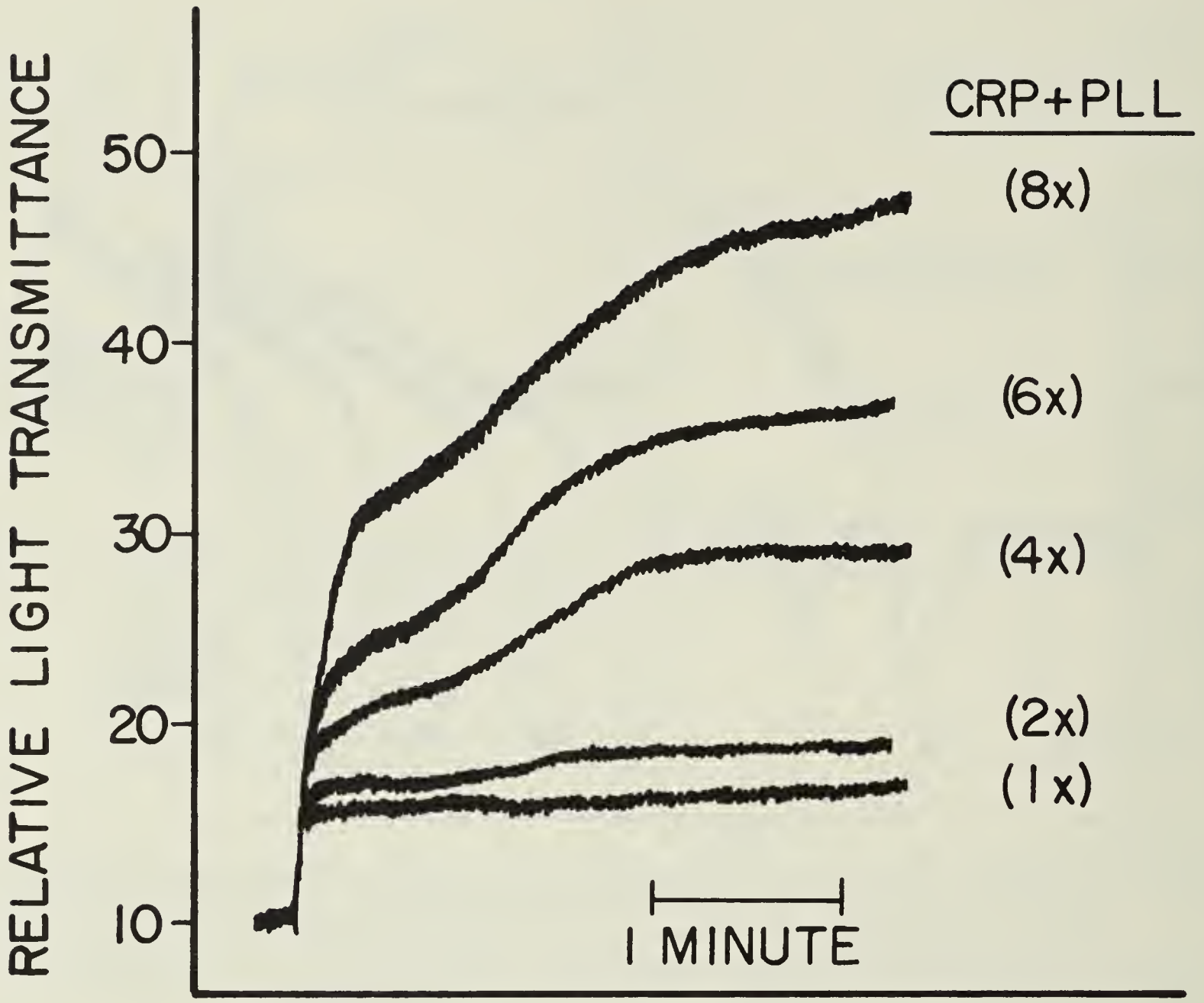

Fig. 12. Aggregation of isolated human platelets by complexes formed between CRP and poly-L-lysine (PLL; 15,000 daltons) at a 70:1 CRP:PLL (w/w) ratio. CRP and PLL were incubated at 37° for 15 min and aliquots of the washed precipitate were brought to appropriate concentrations (parenthesis) in TBS. PLL alone at the concentrations used in these experiments did not induce platelet aggregation.

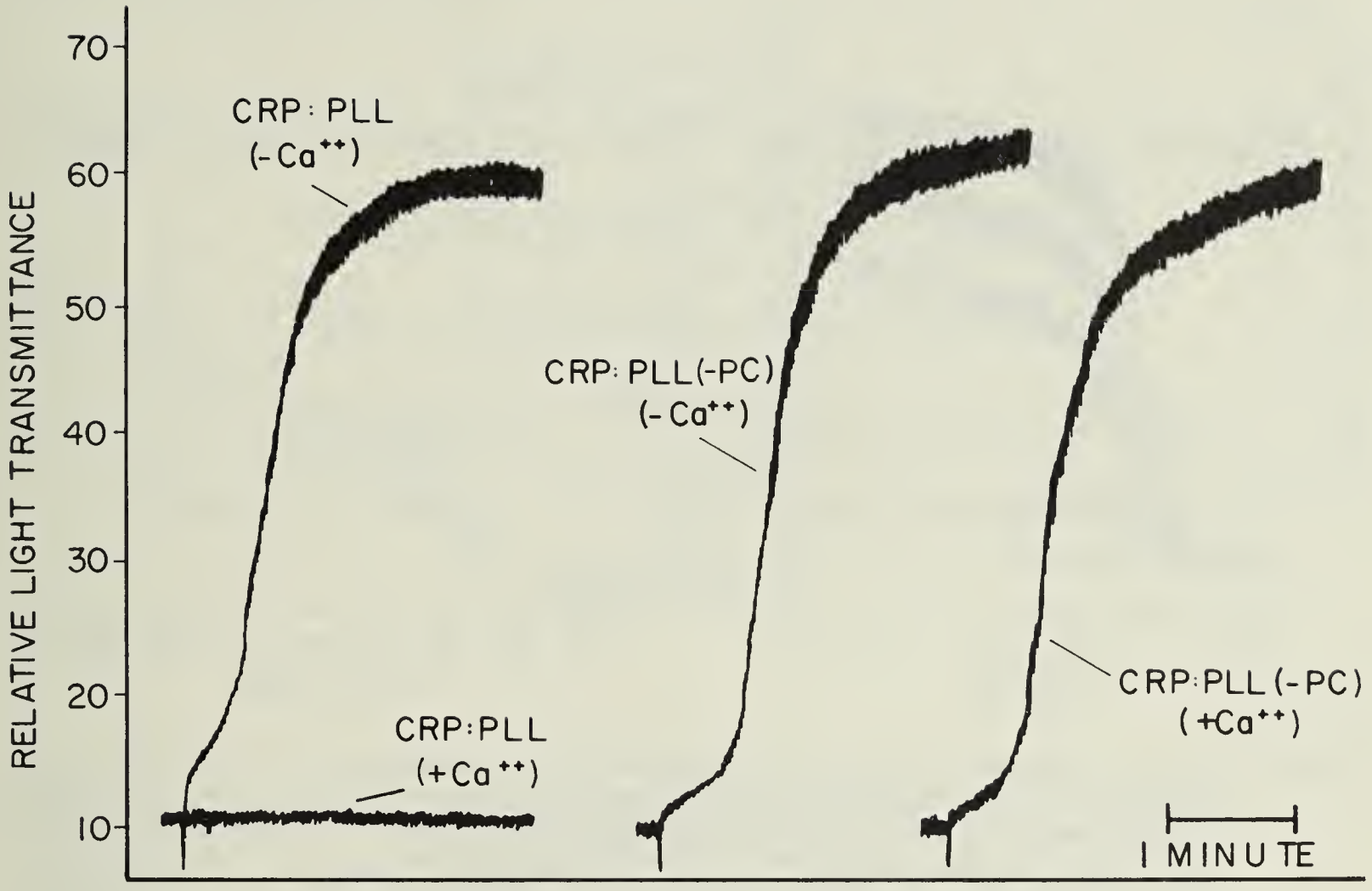

Fig. 13. Effect of the covalent attachment of PC to PLL on the inhibitory influences of Ca^{++} during CRP-poly-L-lysine complex stimulated platelet aggregation. Such attachment negated the depressive influence of calcium ions. (See also Fiedel et al., 1982c).

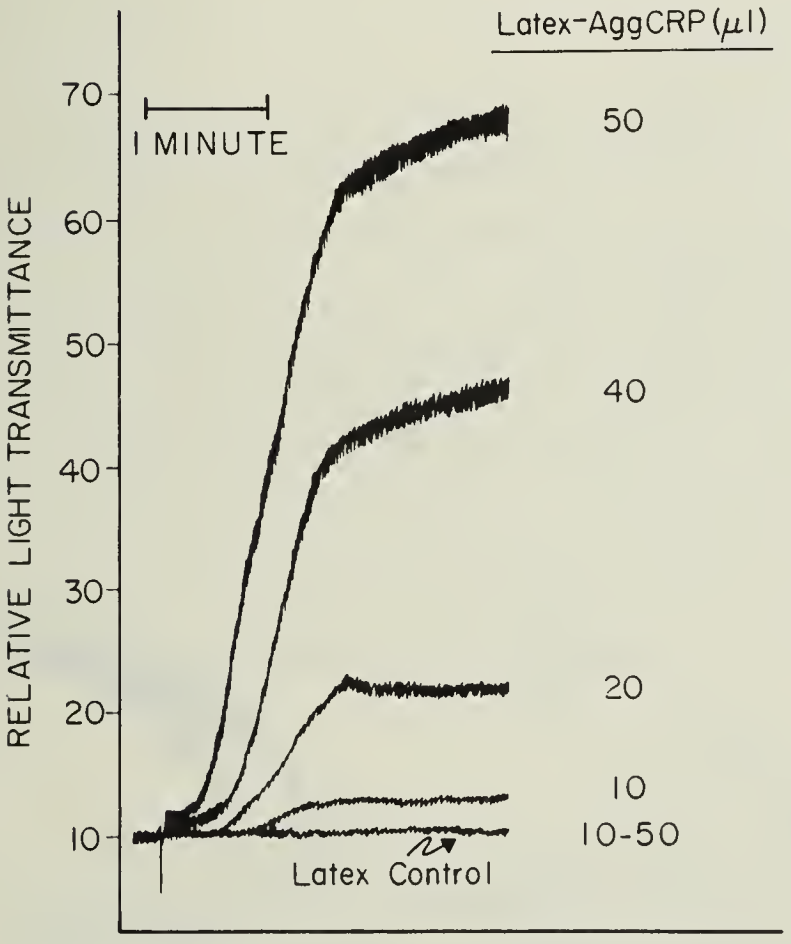

Fig. 14. Activation of isolated platelets by latex-aggregated CRP (Agg CRP). In this experiment each 10 μl addition of latex AggCRP represented approximately 9.6 μg/ml of CRP offered. These values were determined by antigenic analysis of the CRP solution before and following incubation with latex.

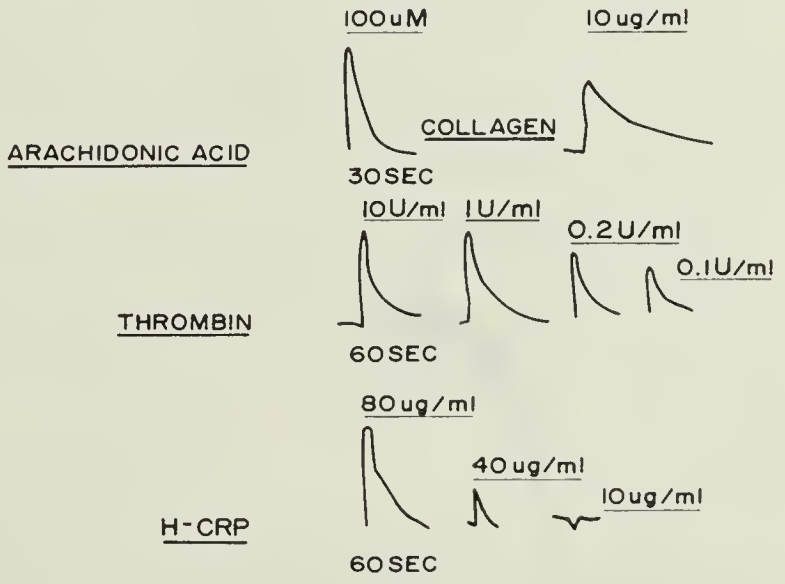

Fig. 15. A comparison of the ability of arachidonic acid, collagen, thrombin or H-CRP to generate TXA_2 activity from platelets. Platelets suspensions were challenged with the concentrations shown and the reaction mixtures assayed. Aortic strips were suspended from transducers at a constant tension of 2 gm/cm and calibrated using a polygraph with recorder.

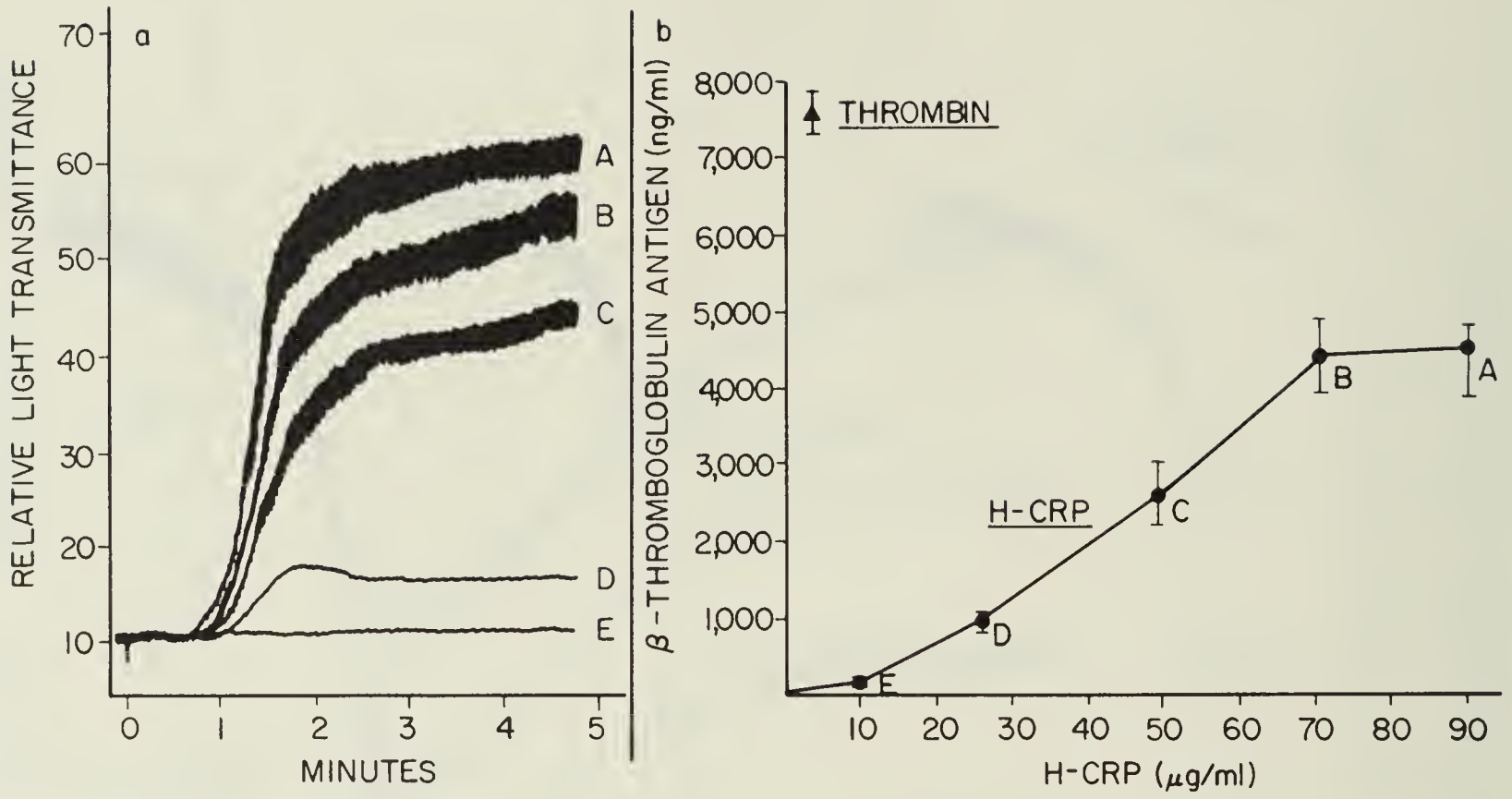

Fig. 16. In (a), the aggregation response of isolated platelets to increasing amounts of H-CRP. In (b), the release of β-thromboglobulin antigen from platelets challenged in (a) using five different H-CRP concentrations marked A-E and corresponding to the H-CRP concentrations used in (a). β-Thromboglobulin antigen was measured using a radioimmunoassay with platelet activating conditions as described in Materials and Methods. For comparison, the β-thromboglobulin antigen released following challenge of platelet suspensions with a concentration of thrombin (▲)yielding a maximal platelet aggregation event is depicted in (b). Data are given as the mean and range of three independent determinations for each H-CRP or thrombin concentration used.

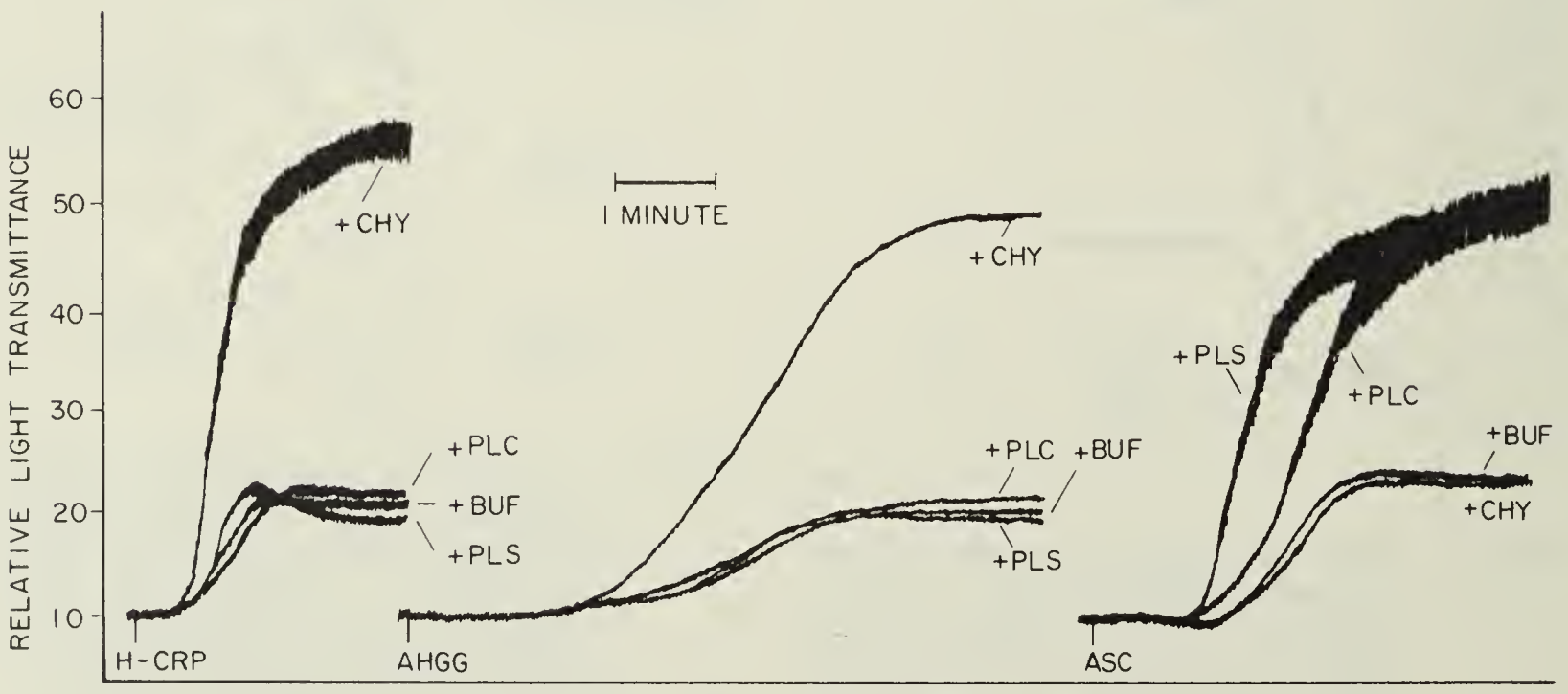

Fig. 17. Effect of enzyme treatment on the platelet response to H-CRP, AHGG and ASC. CHY = chymotrypsin; PLC = phospholipase C and PLS = plasmin.

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Light and Electron Microscopic Examination of *Pleistophora Cepedianae* Putz, Hoffman, and Dunbar (Microsporida: Pleistophoridae) from Gizzard Shad, *Dorosoma Cepedianum* LeSueur

Robert L. Price
Department of Zoology and
Center for Electron Microscopy
Southern Illinois University at Carbondale
Carbondale, Illinois 62901

ABSTRACT

Developmental and mature stages of *Pleistophora cepedianae* from gizzard shad, *Dorosoma cepedianum*, were examined by light and electron microscopy. Cysts were found in connective tissue associated with all portions of the digestive tract as well as in the caudal musculature. Schizonts are initially amorphous multinucleate bodies which elongate resulting in nuclei aligning in a straight row. Ultrastructural characteristics of developmental stages differ from those published for *Pleistophora* in lacking membrane lined channels in the amorphous coat of schizonts and formation of sporoblasts from rosette shaped sporogonial plasmodia. Mature spores average 5.0 μm in length and 2.4 μm in width. Polar filaments have 15 to 17 coils aligned in a single row. Both vesicular and lamellar polaroplast regions are present.

INTRODUCTION

The importance of microsporidians as obligate intracellular parasites has been recognized for many years as evidenced by monographs such as Kudo's (1924) Biologic and Taxonomic Study of the Microsporidia and numerous papers dated

before 1900. Unfortunately, the status of many taxa within the group is unclear as evidenced by the shifting of genera and families in the various taxonomic schemes which have been suggested (Kudo, 1924; Sprague, 1977).

Sprague (1977) characterised the genus *Pleistophora* as an inadequately defined genus that will eventually need to be broken up. He also suggested that since the type species is in fish it will probably remain a genus primarily of fish parasites, while many of the species in invertebrates will eventually go into new genera. Canning and Hazard (1982) subsequently examined several species of *Pleistophora* by electron microscopy and concluded the genus consisted of an assemblage of at least three genera, suggesting further ultrastructural examination of species in vertebrates would result in these remaining in *Pleistophora*, while species in invertebrates may need to be shifted to other genera.

Developmental and mature stages of *Pleistophora cepedianae* were examined by light, and transmission and scanning electron, microscopy. The only previous reports of *P. cepedianae* have been by Putz et al. (1965) who described developmental and mature stages by light microscopy and Price (1982) who discussed prevalence of the parasite in gizzard shad of Carlyle Lake, Illinois.

METHODS AND MATERIALS

Gizzard shad were collected by seine from Carlyle Lake, Clinton County, Illinois, from July to September, 1982 and during July, 1983. Fish were pithed and immediately examined for opaque white cysts characteristic of microsporidian infections. Fresh spores were prepared by the paraffin oil method of Vavra (1964) for measurement of spore size.

Cysts prepared for paraffin embedding were preserved in 10% phosphate buffered formalin, washed in distilled water, dehydrated through a graded ethanol series and embedded in paraffin. Eight micrometer sections were stained with either Harris' hematoxylin or Mallory's modified aniline blue which is specific for collagenic connective tissue (Preece, 1965).

Cysts prepared for transmission electron microscopy (TEM) were preserved in 3% glutaraldehyde prepared with 0.1M cacodylate buffer (pH 7.2), post fixed in 2% aqueous osmium tetroxide, washed in distilled water, and dehydrated through a graded ethanol series. Infiltration and embedding were performed in vacuo with either Spurr's low viscosity embedding media (Spurr, 1969) or the medium formula of LR White embedding media by the method of Murphy and Price (1983).

Two micrometer sections of cysts prepared in Spurr's and LR White were obtained on glass knives and stained with 0.1% toluidine blue prepared with 1.0% borax. Sections for TEM were obtained on diamond knives on a Reichert OM-U2 ultramicrotome and stained with 2% aqueous uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Examination was in the Hitachi H500H at 75 kV.

Cysts prepared for scanning electron microscopy (SEM) were preserved as for TEM, critical point dried in a Samdri 790, mounted on aluminum stubs using Scotch 666 double sticky tape and silver conducting paint, and coated with gold palladium in a Hummer V sputter coater. Examination was in a Cambridge Mark IIA at 10 and 20 kV.

Measurement of polar filament angle was performed by the method of Burges et al. (1974) using median longitudinal sections of mature spores.

RESULTS

Cysts were found in connective tissue associated with all portions of the digestive tract as well as the caudal musculature (Fig. 1). By light microscopy spores appeared either slightly tapered at the anterior end or oval. Fresh spores averaged 5.0 μm in length and 2.4 μm in width. Little information concerning internal organelles was visible in fresh and stained smears due to small spore size and the chitinous endospore. Pansporablasts contain more than 16, and usually more than 30, developing spores. Pansporoblast size varied with number of spores developing within.

Two micrometer sections of young cysts show a thin cyst wall with a developmental zone of schizonts, sporonts, and pansporoblasts immediately interior (Fig. 2). Sporoblasts separate from a rosette shaped sporogonial plasmodium within a pansporoblast membrane (Fig. 3). Greater than 20 spores were frequently seen in a 2 μm section of a pansporoblast (Fig. 4). Width of the developmental zone decreases as cysts matured until finally spores lined the cyst wall (Fig. 5). Host fibroblasts line the thin cyst wall in young cysts (Fig. 2) and are replaced by a wide zone of collagen fibers in mature cysts (Fig. 5).

Transmission electron micrographs through the wall of a young cyst shows a narrow zone of host cell organelles with numerous mitochondria and fragments of host nuclei. Inside the zone of host organelles are developmental stages of the parasite (Fig. 6).

Schizonts are first seen as amorphous multinucleate bodies. Mitotic spindles in dividing nuclei were occasionally seen. Zones of endoplasmic reticulum were usually adjacent to nuclei (Fig. 7). As schizonts elongate nuclei arrange in a straight row and the limiting membrane becomes thicker in preparation of pansporoblast membrane formation (Fig. 8). As many as twelve nuclei were observed in a thin section.

As the schizont wall thickens vesicles form (Fig. 9) which continue expanding to form the pansporoblast membrane (Fig. 10). As the pansporoblast membrane develops schizonts divide to form sporonts. Sporogonial plasmodia form rosette shaped structures (Fig. 11) from which sporoblasts divide. Sporoblasts retained within a pansporoblast membrane elongate and nuclei migrate to one end. Endoplasmic reticulum orients along the length of the sporoblast (Fig. 12).

Polar filament formation is closely associated with structures Vavra (1965) identifies as "primitive" Golgi apparati (Fig. 13). The filament is formed in several segments which coalesce and align along the inside periphery of the late sporoblast. The endospore and exospore are both well formed before the filament completes alignment and polaroplast areas are differentiated (Fig. 14).

Mature spores have 15 to 17 coils in the polar filament which are aligned in a single straight row along the inside periphery of the spore (Fig. 15). Angle of anterior coils averages 80° while that of posterior coils averages 73.5°. The manubrium of the polar filament courses through the vesicular and lamellar polaroplast ending in a terminal or slightly subterminal anchoring disc (Fig. 16). Higher magnifications of filament coils show they consist of six to seven concentric electron dense and electron transparent rings (Fig. 17). The posterior vacuole occupies up to one half of the posterior spore and contains dense flocculent material. Spore walls consist of an exospore 32.4A in thickness and endospore 48.6A thick except over the anchoring disc where it thins to 14A (Fig. 15).

Spores remain encased in pansporoblast membranes until mature. Spore surfaces are smooth with no apparent ornamentation (Fig. 18).

DISCUSSION

Importance of electron microscopy in the taxonomy of the genus *Pleistophora* has taken on added significance with the attempt by Canning and Hazard (1982) to split the genus into at least three genera, *Pleistophora*, *Vavraia*, and *Polydispyrenia*. *Polydispyrenia* can easily be distinguished from *Pleistophora* and *Vavraia* by diplokarya in young sporonts and formation of the sporophorous vesicle wall (pansporoblast membrane) at the onset of sporogony. Sporophorous vesicles in both *Pleistophora* and *Vavraia* begin formation during schizogony and are derived from amorphous secretions laid down external to the plasmalemma. In *Pleistophora* the amorphous material is permeated by membrane lined channels which are absent in *Vavraia*.

Sporulation in *Pleistophora* is polysporous with stepwise division through multinucleate segments into many uninucleate sporoblasts. Both microspores and macrospores are found, although macrospores are rare. Sporulation in *Vavraia* is by multiple fission of a rosette formation resulting in uninucleate sporoblasts. Only microspores are found.

Lack of membrane lined channels in the amorphous coat of schizonts, and formation of sporoblasts by multiple fission of a rosette, may indicate *P. cepedianae* should be shifted to the genus *Vavraia*. However, *Pleistophora* sp. from the stoneroller, *Campostoma anomalum*, also lacks membrane lined channels in the amorphous coat of schizonts, but does form sporoblasts by stepwise division through multinucleate segments (Price, 1983). I hesitate to transfer *P. cepedianae* to *Vavraia* since it does occur in a vertebrate host. After ultrastructural examination and interpretation of developmental stages from several more species of *Pleistophora* in both vertebrates and invertebrates a clearer distinction of genera may be possible.

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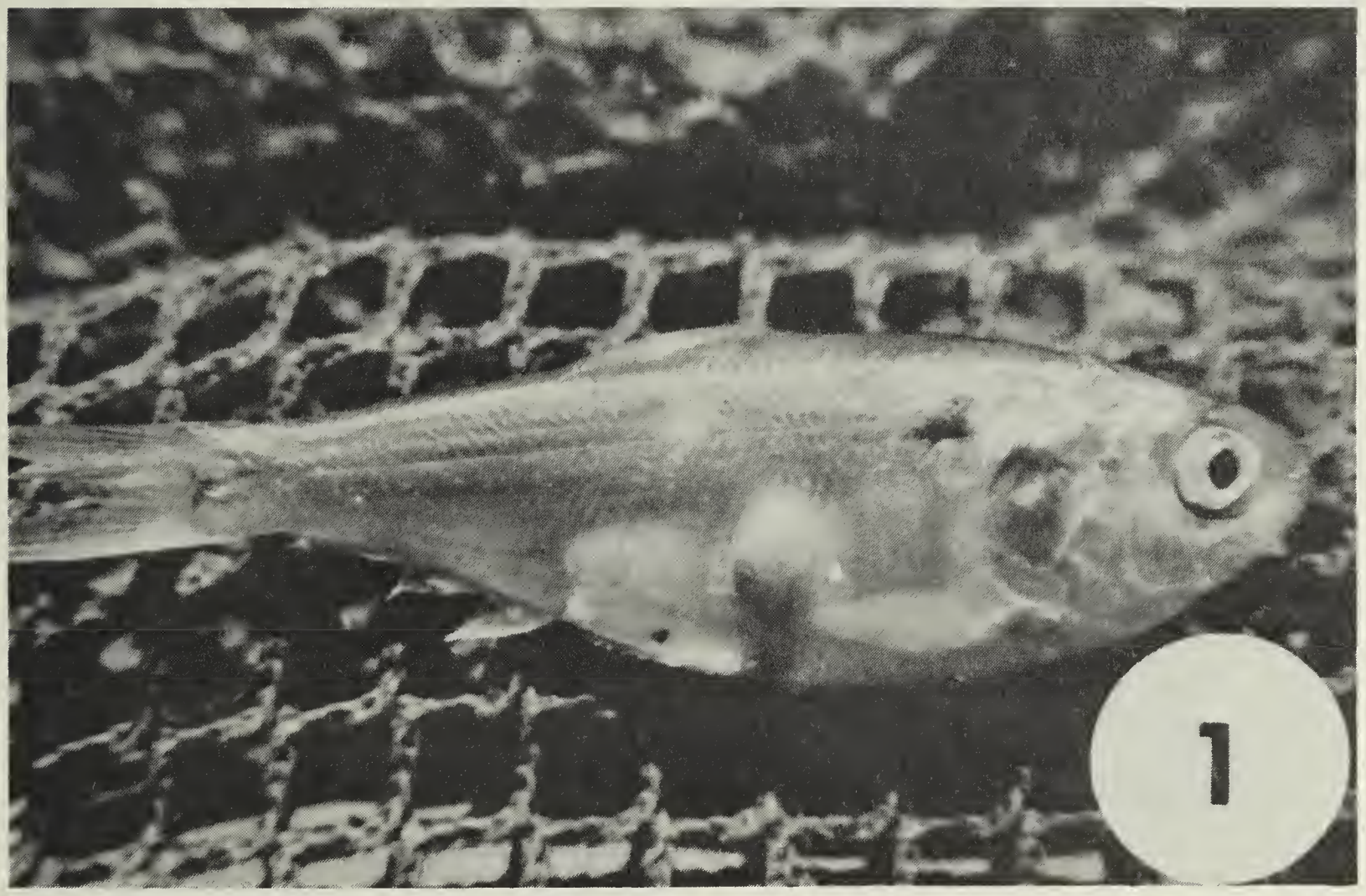
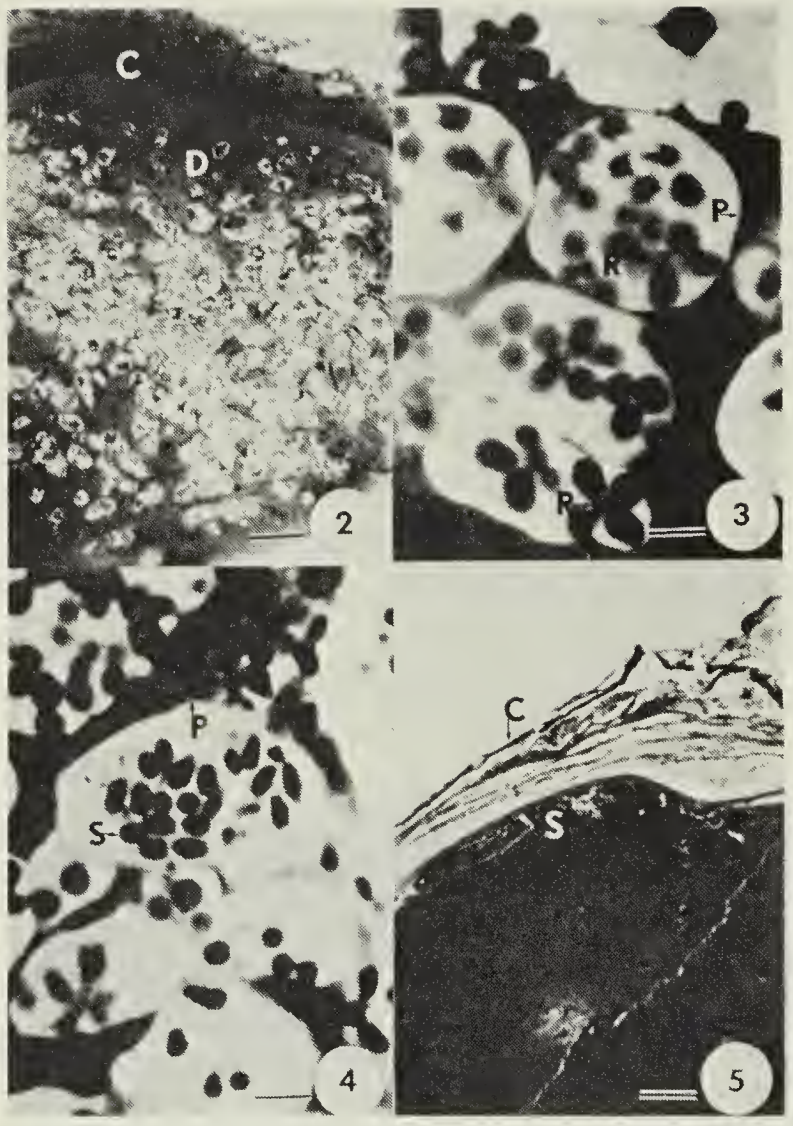


Fig. 1. Gizzard shad, *Dorosoma cepedianum*, with large *Pleistophora cepedianae* cyst protruding from the body cavity.



Figs. 2-4. Two micrometer sections through developing *Pleistophora cepedianae* cyst. 2. Section through thin cyst wall (C) and zone of developing parasites (D). Scale = 50 μm. 3. Pansporoblasts (P) containing rosettes (R) of developing sporonts. Scale = 8 μm. 4. Pansporoblast (P) with at least 20 spores. Scale = 8 μm.

Fig. 5. Eight micrometer section through mature cyst. Spores (S) line cyst wall which is composed of a thick zone of collagen fibers (C). Scale = 50 μm.

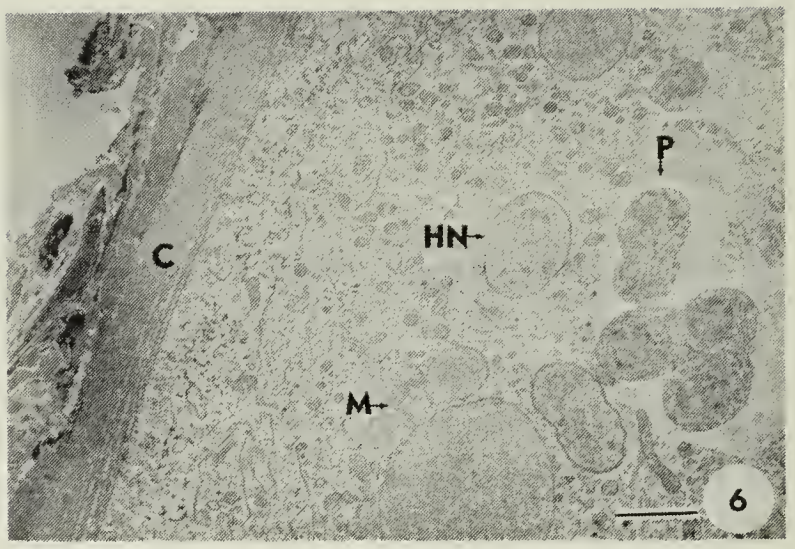
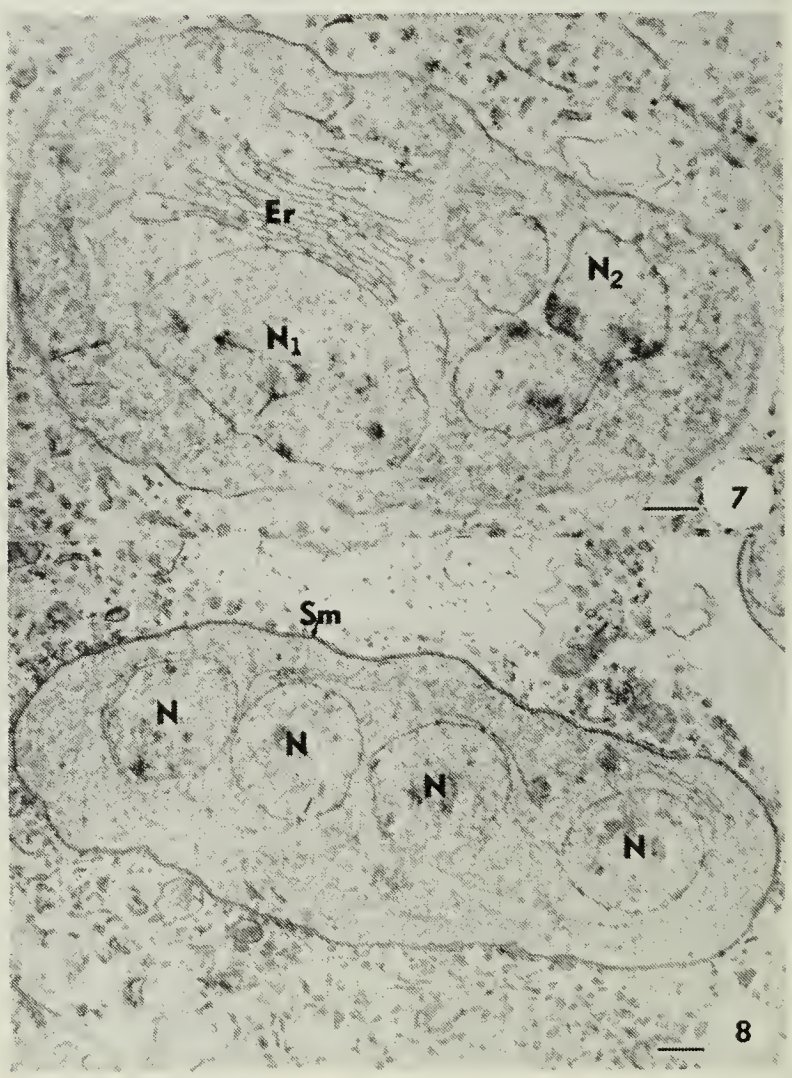


Fig. 6. Transmission electron micrograph through cyst wall of developing *Pleistophora cepedianae* cyst. Inside cyst wall (C) is zone of host cell organelles, including numerous mitochondria (M) and nuclei (HN). Early developmental stages of the parasite (P) are inside the zone of host organelles. Scale = 5 μm.



Figs. 7-8. Transmission electron micrographs of *Pleistophora cepedianae* schizonts. 7. Amorphous multinucleate schizont with dividing nucleus (N_2) and endoplasmic reticulum (Er) closely associated with nuclei. Scale = 1 μm. 8. Elongate schizont with nuclei (N) in a straight row. The schizont membrane (Sm) is thicker than that seen in Figure 7 in preparation of pansporoblast membrane formation. Scale = 1 μm.

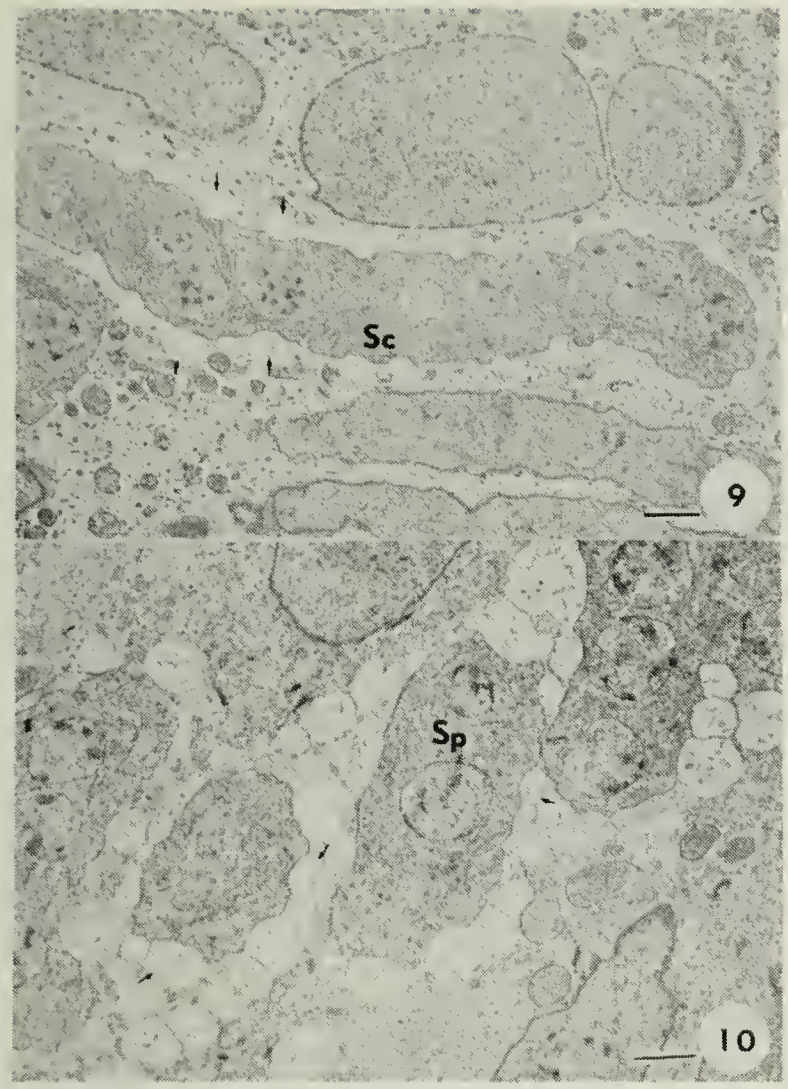


Fig. 9. Wall of schizont (Sc) forming vesicles (arrows) initiating pansporoblast membrane formation. Scale = 3 μm.

Fig. 10. Vesicles (arrows) have expanded and schizont has divided to form sporonts (Sp). Scale = 3 μm.

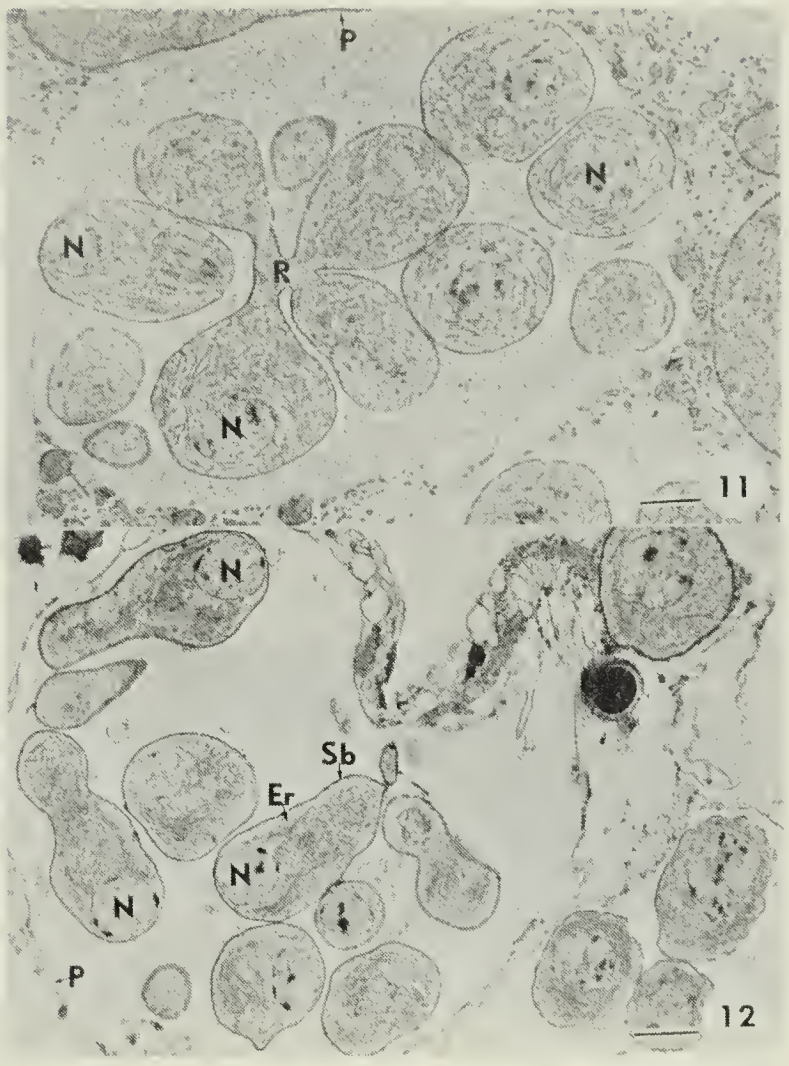
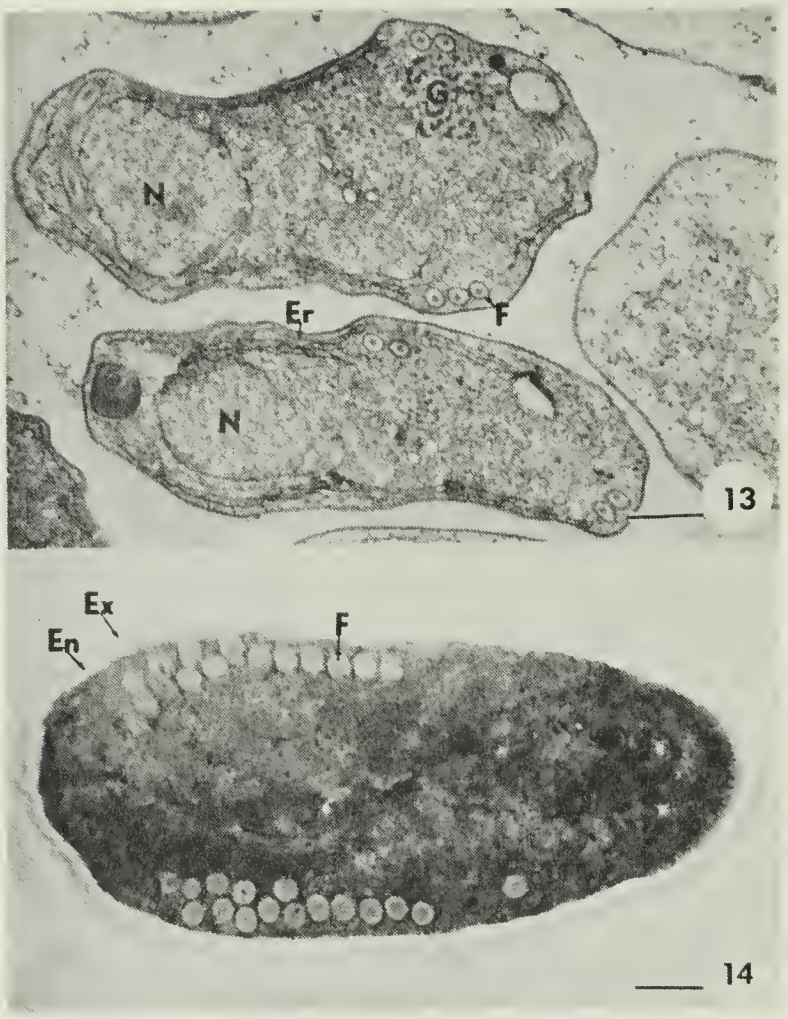
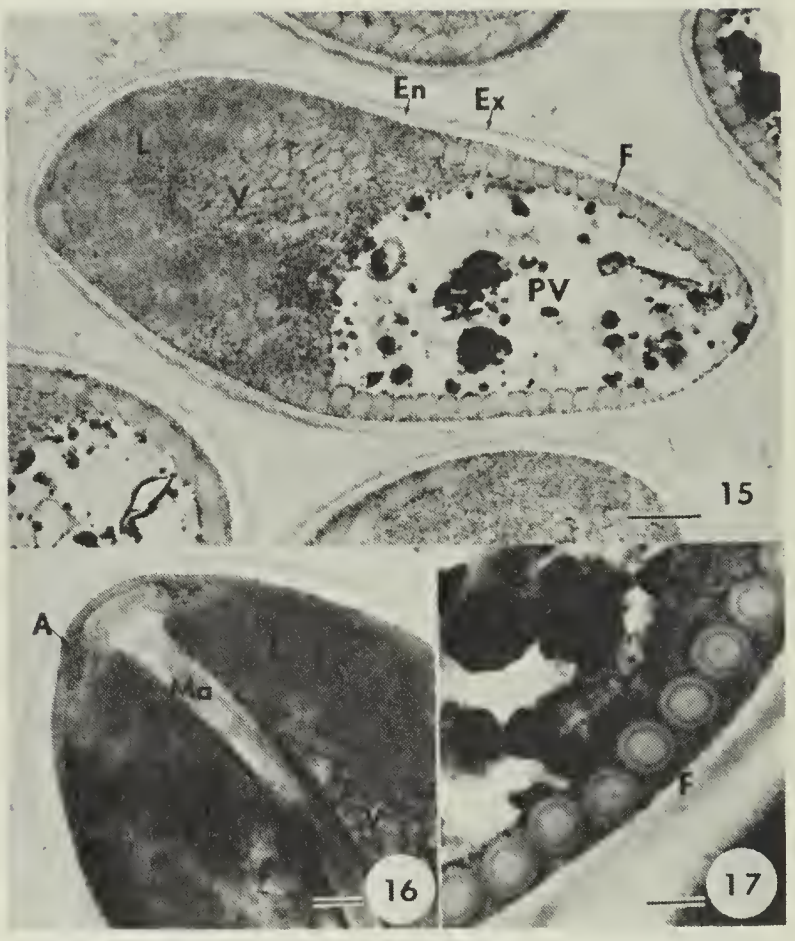


Fig. 11. Rosette shaped sporogonial plasmodium (R) dividing to form unincleate (N) sporoblasts within pansporoblast membrane (P). Scale = 3 μm.

Fig. 12. Elongate sporoblasts (Sb) with nuclei (N) at one end and endoplasmic reticulum oriented along length. Scale = 3 μm.



Figs. 13-14. Late sporoblasts of *Pleistophora cepedianae*. 13. Development of polar filament in close association with "primitive" Golgi apparatus (G). Scale = 1 μm. 14. Late sporoblast with polar filament (F) aligning around periphery as endospore (En) and exospore (Ex) develop. Scale = 0.5 μm.



Figs. 15-17. Mature *Pleistophora cepedianae* spores. 15. Section through spore showing arrangement of lamellar (L) and vesicular (V) polaroplast regions, posterior vacuole (PV), polar filament (F), and spore wall (Ex, En). Scale = 0.5 μm. 16. Anterior end of spore showing arrangement of anchoring disc (A), manubrium of polar filament (Ma), and lamellar and vesicular polaroplast regions. Scale = 0.05 μm. 17. High magnification illustrating 6 to 7 concentric rings of polar filament. Scale = 0.05 μm.

Fig. 18. Scanning electron micrograph of mature *Pleistophora cepedianae* spores encased in pansporoblast membranes. Scale = 5 μm.

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Hyperthermia and Radiation Induced Chromosome Loss in Anoxia in Drosophila

Sidney Mittler
Department of Biological Sciences
Northern Illinois University
DeKalb, Illinois 60115 USA

ABSTRACT

Male Drosophila *Oregon R*, and the mutagen sensitive strains, *y mei-9*^{a} and *y mei-9*^{a}*mei-41*^{D5} all with $B^{s}Yy^{+}$ were subjected to 42°C in anoxia and then irradiated with 2000 R of x-rays in anoxia. The hyperthermia treatment significantly increased the radiation induced loss of chromosomes in *Oregon R* in 5 out of 12 broods (two in CO_2 and three in N_2 series), but none in the broods of *y mei-9*^{a} and only in one of *y mei-9*^{a}*mei-41*^{D5}. The failure of excision repair deficient strains to be affected by hyperthermia in anoxia in increasing radiation induced genetic damage may signify that hyperthermia may decrease the efficiency of repair enzymes.

INTRODUCTION

The influence of hyperthermia on the enhancement of radiation induced damage has rapidly led to numerous investigations utilizing heat treatment in cancer therapy. The mechanisms of the radiation sensitization by the heat treatment is not fully understood. The impairment of repair enzymes by the hyperthermia has been suggested for the increase in damage to DNA and chromosomes (Dewey, Sapareto and Betten, 1977; Dube, Seal and Loeb, 1977; Corry, Robinson and Getz, 1977; and Mittler, 1980, 1981). Hyperthermia increased radiation induced chromosome loss, recessive and dominant lethals in *Drosophila melanogaster* (Mittler, 1979a, 1979b). Mittler (1981) utilized mutagen sensitive stocks which were deficient in several DNA repair pathways and reported that the *mei-9*^{a} strain deficient in excision repair did not respond to the increase in radiation induced chromosome loss as a result of pretreatment by hyperthermia. This may indicate that hyperthermia may affect the excision repair system. The investigation presented here was to study whether the hyperthermia would influence radiation induced chromosome loss in spermatogenesis of wild type and mutagen sensitive strains (with defective repair pathways) if the hyperthermia treatment and radiation were given in anoxia.

MATERIALS AND METHODS

Drosophila melanogaster males containing a Y chromosome with translocation of y^+ and B^s of the mutagen-sensitive strain *y mei-9*^{a}, the double mutants *y mei-9*^{a}*mei-41*^{D5}, and the wild type *Oregon R* stock were used to determine whether hyperthermia would have an effect on radiation induced chromosome loss in anoxia. Boyd and Setlow (1976) have proposed four tentative classes of DNA repair pathways for mutagen-sensitive Drosophila mutants. *mei-41*^{A1} was assigned to Class 1 (postreplication repair deficient, meiotic defective, residual postreplication repair not strongly sensitive to caffeine). *mei-9*^{a} was assigned to Class 3 in which the mutants are postreplication proficient with DNA metabolism partially caffeine sensitive and *mei-9*^{a} is also deficient in excision repair (Boyd, Golino and Setlow, 1971). The males were kept in anoxia either in N_2 or CO_2 for 12 minutes in a circular leucite container machined so that the two halves were grooved to contain four No. 000 gelatin capsules and designed to permit gases to pass. One day old adult males were placed in the capsules that had holes at either end and put into a leucite container with 500 ml/min CO_2, N_2 or air flowing through and kept submerged in a water bath at 42°C for 7 minutes for the hyperthermia exposure. With the gas still flowing the leucite container was lifted out of the water bath and placed in a G.E. Maximar III unit and was exposed to 2000 R of x-rays within 30 seconds at 250 KV, 15 ma, 32 cm at 419 R/min. The time for the hyperthermia plus radiation treatments totaled about 12 minutes and about 90% of the flies survived this treatment. Although adult Drosophila can survive 1 hour in anoxia and a half hour at 42°C in air, the combination of hyperthermia and anoxia sharply reduced the time that adult males could survive. The controls were kept in anoxia for 12 minutes, along with those groups of flies that received only hyperthermia or radiation. The males were then mated to y^2w^{sp} (1 ♂ : 3♀♀) and transferred every two days to a virgin group of females for 6 or 7 two day broods. This brooding tended to separate the effects on the various stages of spermatogenesis at the time of irradiation (Auerbach, 1954) in that offspring produced in 0-2 day brood represented mature or almost mature spermotozoa, 2-4 day brood represented late spermatids, while early spermatids by 4-6 day brood, 6-8 day broods represented spermatocytes, 8-10, 10-12, 12-14 day broods represented spermatogonia at the time of irradiation (Mittler, 1966).

The loss of the X or Y chromosomes in spermatogenesis were detected in the F_1 offspring by appearance of exceptional phenotypes. The loss of the X or Y resulted in XO males, y^2w^{sp}, yellow white speckled eye; the loss of the B^s translocation in a round eye wild body male; and the loss of the y^+ in a yellow body Bar eye male. Paternal nondisjunction was detected by the appearance of a wild body, Bar eye female. Each series was rerun at least three times and data pooled, with more males used in the radiation and the hyperthermia plus radiation experiments because of induced sterility.

RESULTS

The data obtained by hyperthermia and radiation in N_2 for *Oregon R*, *y mei-9*^{a} and *y mei-9*^{a}*mei-41*^{D5} strains are presented in Figure 1 and the data for radiation and hyperthermia in air and CO_2 for these strains are plotted in Figure 2. The double mutagen-sensitive strain y *mei-9*^{a}*mei-41*^{D5} is the most sensitive of the three to radiation induced chromosome loss in N_2 (Figure 1c) and in air and CO_2 (Figure

2c). The 2000 R of x-rays induced sterility in the males of both of the mutagen sensitive strains in the spermatogonia represented by the 8-10 and 10-12 day broods.

In the wild type strain *Oregon R*, the hyperthermia significantly increased the radiation induced chromosome loss in air in the spermatogonia, 8-10 and 10-12 day broods (Figure 2a). With respect to irradiation in CO_2, chromosome loss was significantly increased by hyperthermia treatment in broods 6-8 and 10-12 days (Figure 2a). The hyperthermia treatment significantly increased the radiation chromosome loss in N_2 broods 4-6, 8-10, and 10-12 days in *Oregon R* (Figure 1a).

The hyperthermia treatment in air, N_2 or CO_2 did not significantly increase the radiation induced chromosome loss in any of the stages of spermatogenesis of the mutant *y mei-9^a*. In the double mutant *y mei-9^{a}mei-41^{D5}* the hyperthermia treatment in the air series only increased the radiation induced chromosome loss in one brood, 0-2 days, which represented the mature or almost mature spermatozoa. There was no significant increase by the hyperthermia treatment in radiation induced chromosome loss in any of the broods in the CO_2 and N_2 series in *y mei-9^{a}mei-41^{D5}* stock. The 42°C treatment for 7 minutes did not significantly induce chromosome loss in any of the broods of the three stocks studied when given in CO_2 or N_2.

DISCUSSION

The radiation treatment of repair deficient mutants with 2000 R of x-rays in air and in anoxia severely reduced the number of offspring in those broods, 8-10, 10-12, 12-14 days which represented spermatogonial cells at the time of radiation. This did not occur in the spermatogonial cells of the wild type *Oregon R*. The hyperthermia treatment in anoxia (but not in air) reduced the number of spermatogonial cells of the repair deficient mutants. The reduction in offspring was not as severe when the heat treatment was given in air. There was no difference in the radiation induced loss of chromosome whether CO_2 or N_2 was used for the method of anoxia in the three strains.

The pretreatment with 42°C and irradiation of the wild *Oregon R* significantly increased the loss of chromosomes in spermatogenesis induced by radiation in 5 out of 12 possible broods in anoxia (two in CO_2 and three in the N_2 series). Similar hyperthermia treatments in anoxia did not increase radiation induced chromosome loss in any of the 22 broods produced by the two repair deficient strains. There were also no significant increases produced by hyperthermia on radiation induced chromosome loss in *y mei-9^a*, the excision repair deficient strain, in air. This is in agreement with the report by Mittler (1981) that the mutant, *y mei-9^a* did not respond to the hyperthermia radiosensitization in air. Since hyperthermia increased radiation induced chromosome loss in mutants of other repair pathways and in wild type Drosophila, this led to a hypothesis that the hyperthermia treatment could not impair an already inherited mutant block. The failure of the repair deficient strains to respond to hyperthermia radiosensitization in anoxia also tends to strengthen the hypothesis that hyperthermia may inactivate the repair enzymes. There was also a possibility that excision repair mutants were so sensitive to radiation that this sensitivity overrode whatever the effect of hyperthermia would add to the increase in radiation induced loss.

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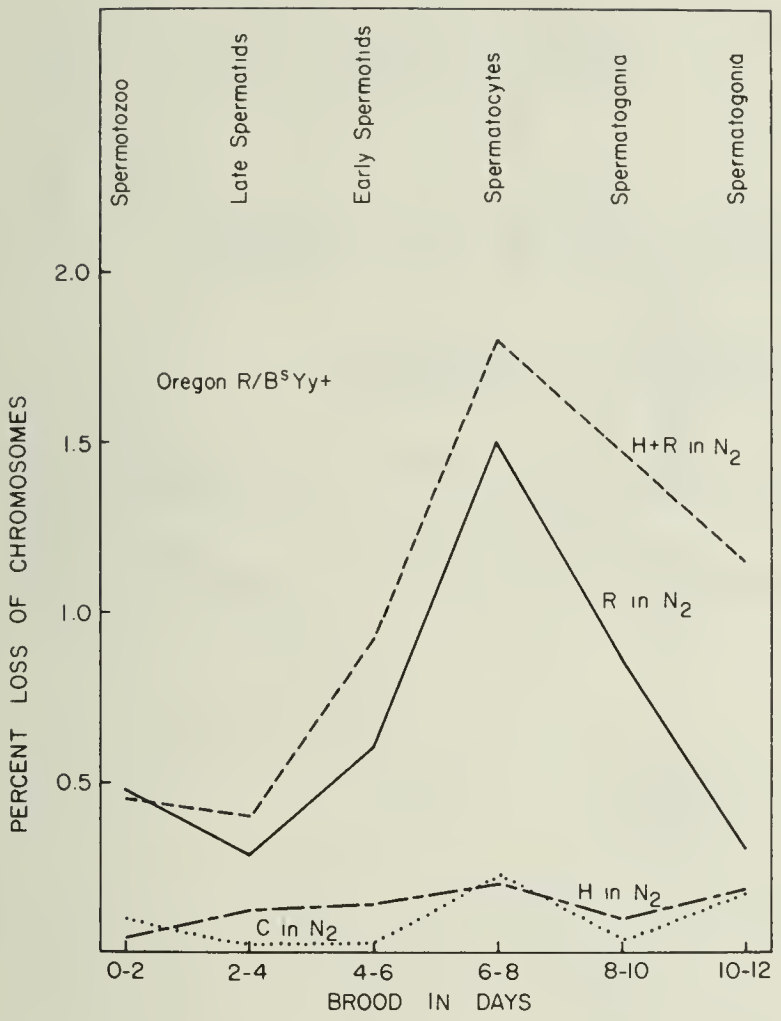

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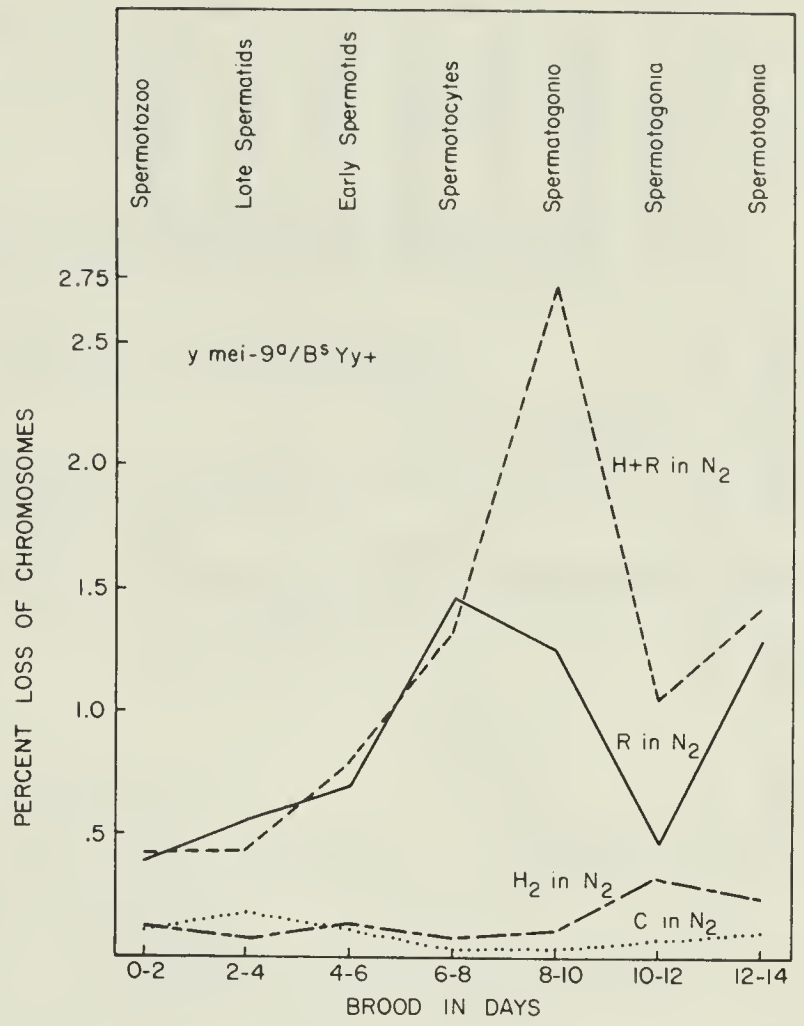

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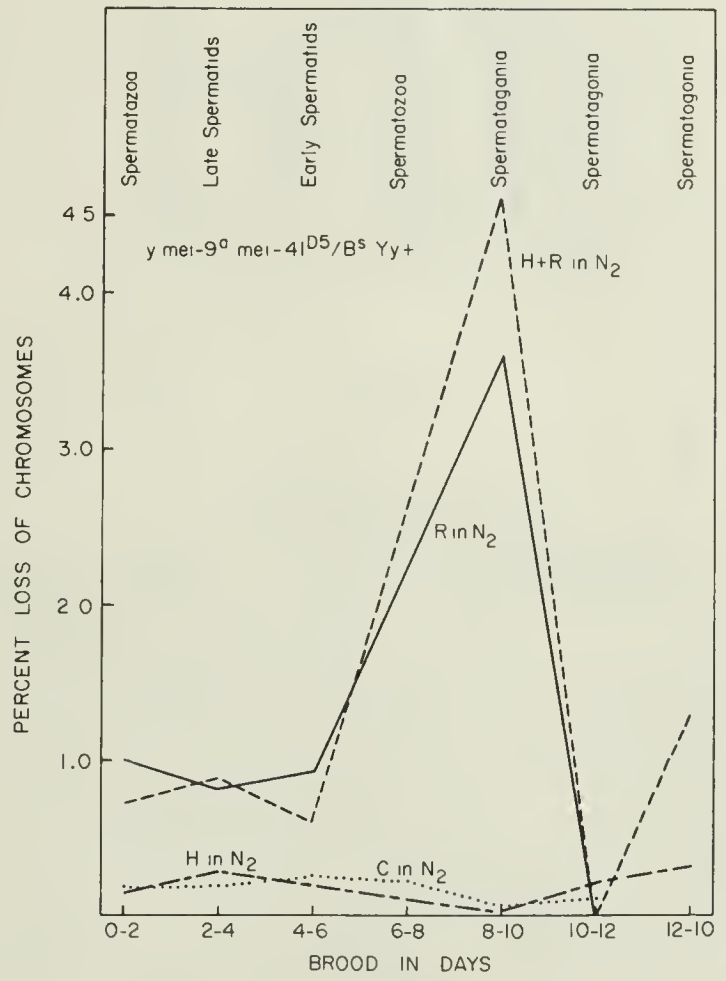

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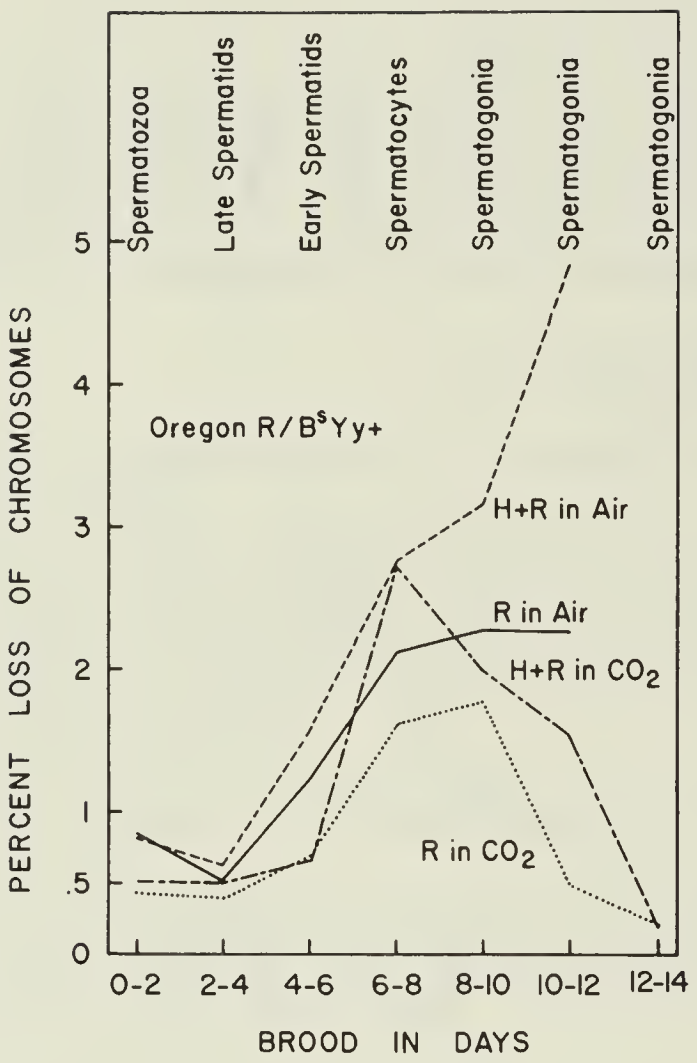
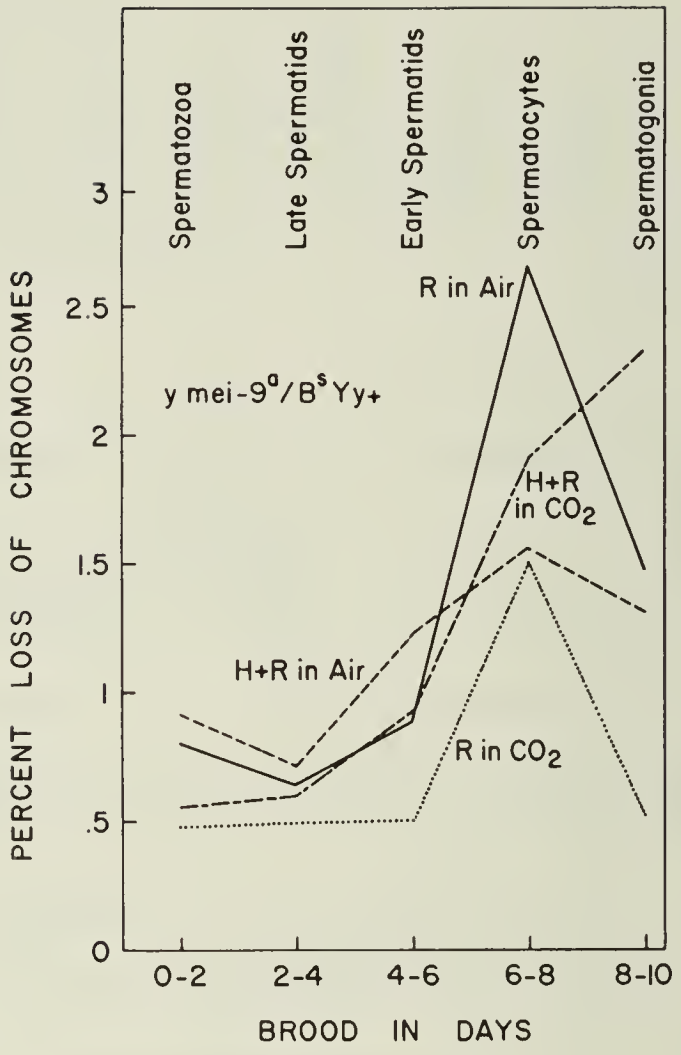
Fig. 1. The effect of hyperthermia upon radiation induced loss of chromosomes in spermatogenesis of (a) Oregon $R/B^{s}Yy^{+}$, (b) y mei-$9^{a}/B^{s}Yy^{+}$, and (c) y mei-9^{a}mei-$41^{D5}/B^{s}Yy^{+}$ when radiation of 2000R of x-rays and hyperthermia of 42°C were given in N_2.





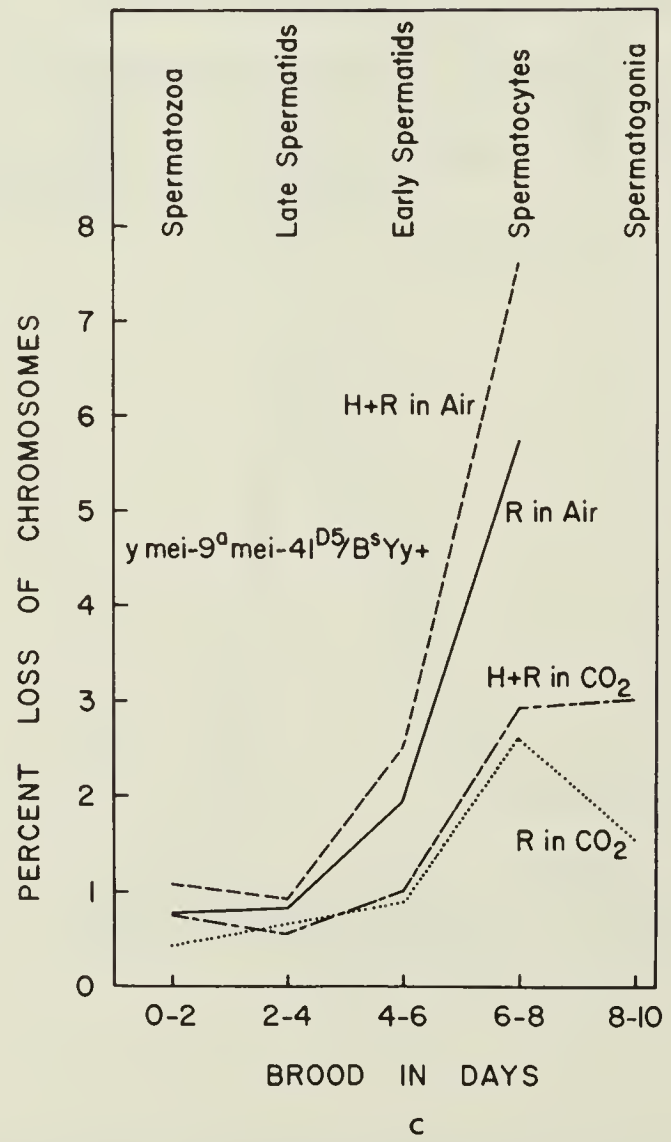


Fig. 2. The effect of hyperthermia upon radiation induced loss of chromosomes in spermatogenesis of (a) Oregon R/B^sYy^+, (b) y mei-$9^a/B^sYy^+$, and (c) y mei-9^amei-$41^{D5}/B^sYy^+$ when radiation of 2000R of x-rays and hyperthermia of 42°C were given in air or CO_2.

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Effects of Flooding on Silver Maple (*Acer saccharinum* L.) and American Sycamore (*Platanus occidentalis* L.) Seedlings: Implications for Biomass Production

David L. Peterson
USDA Forest Service
Pacific Southwest Forest and Range Experiment Station
Riverside Fire Laboratory
4955 Canyon Crest Dr.
Riverside, CA 92507
and
Gary L. Rolfe
Department of Forestry
University of Illinois
110 Mumford Hall
1301 West Gregory Drive
Urbana, Illinois 61801

ABSTRACT

Silver maple and American sycamore were compared to determine their suitability for use in biomass plantations located on flooded sites. One-year old nursery stock of each species was subjected to various periods of inundation: early, late, and a combination of early and late in the growing season. Treatments were compared for leaf weight, stem weight, root weight, total weight, root weight/aboveground weight, and height. Biomass increment (final weight/initial weight) was evaluated for aboveground and belowground components. Early flooding caused greater reductions in the growth of silver maple than of sycamore. Late flooding caused slightly greater reductions in the growth of sycamore than of silver maple. Analysis of biomass increment data suggests that both species have the capacity to recover physiologically and produce substantial amounts of aboveground biomass following all flooding treatments except an early flood of long duration. Silver maple and sycamore appear to be equally suitable for use in intensive biomass production on sites which are periodically flooded.

INTRODUCTION

The potential for increasing energy supplies in the United States by increased use of different types of plant biomass has been recognized for some time (Burwell, 1978). Fossil fuel consumption can be supplemented by the use of biomass fuels at power plants as well as at the level of the individual consumer. It has been demonstrated that the conversion of solar energy for biomass fuel production can be integrated with conventional agricultural and forestry operations (Lipinsky, 1978: Sirén, 1982).

It has been shown that various tree species can be used for biomass production in closely spaced plantations (Szego and Kemp, 1973; Wittwer, et. al., 1978). The most successful production of woody biomass has been achieved with fast-growing hardwood species which are native to floodplain environments (Baker and Blackmon, 1977; Harrington, et. al, 1979; Tuskan and De la Cruz, 1982).

Most experimental woody biomass plantations have been located on well-drained sites which are marginal for agricultural use. In the Mid-west, many poorly-drained sites in floodplains are also marginal for agriculture because of frequent flooding. These sites may be appropriate for biomass plantations which use fast-growing floodplain species, because these species are adapted to periodic flooding. Many floodplain soils are suitable for closely-spaced plantings because they have a high nutrient content (Peterson and Rolfe, 1982) and rarely have soil moisture low enough to induce physiological stress in woody plants.

The selection of appropriate species is critical if flooded sites are to be used for woody biomass production. There is a wide variety of native hardwood species which are potential candidates because of their fast growth rate and tolerance of flooding. There have been many studies of the relative growth of hardwood species under various flooding regimes (Hosner, 1960; Hosner and Boyce, 1962; Dickson, et. al., 1965; Hook and Brown, 1973; Baker, 1977). It is difficult to infer which species would be suitable for biomass plantings, however, because of the wide range of flooding treatments and tree age classes which were used. In addition, most of these studies focused on survival rates rather than measurements of biomass production.

Most of the studies cited above rate silver maple and American sycamore as highly tolerant to flooding. These species are native to Illinois floodplains and are both commercially available in large quantities as bare root stock suitable for mechanical tree planters. We investigated the flood tolerance of 1-year old planting stock of these species by simulating various flooding regimes which could occur in the field. The suitability of silver maple and sycamore for biomass production in flooded environments was assessed by analyzing a wide range of growth characteristics.

METHODS

One-year old bare root nursery stock of silver maple and American sycamore were obtained from a commercial nursery in northern Ohio. The seedlings were originally germinated from seed collected from a floodplain site near the nursery. All nursery stock was wrapped in sphagnum moss and stored at 4°C prior to planting.

Seedlings were planted on May 6, 1980, in plastic pots (20 cm diameter × 20 cm depth) containing potting soil composed of 3 parts silt loam field soil, 1 part sand, 1 part peat moss, and 1 part "Perlite". Seedlings were planted with each stem

situated at its estimated original level with respect to the soil surface. All plants were placed in a shadehouse at 80% full sunlight and were protected from inputs of rainfall. Prior to establishment of treatments, plants were watered as necessary to keep the soil moist but not waterlogged.

Wet weight was measured for individuals of each species prior to planting. Dry weight/wet weight ratios were determined for each species by measuring the wet weight and dry weight (after drying at 80°C for 48 hr) of 20 individuals. Separate ratios were determined for aboveground and belowground biomass for each species.

All plants leafed out within 2 weeks after planting. Treatments were initiated on June 9, with eight individuals in each treatment and control group. Flooded plants were completely inundated in tanks with continuously circulating water. Control plants and nonflooded treatment plants were watered as necessary to keep the soil moist but not waterlogged. A control (nonflooded) group and 9 flooding treatments were established for each species. Treatments were various periods of early (E), late (L), and early and late (EL) periods of flooding. Duration of flooding within each of these groups was 10, 20, and 40 days as shown in Fig. 1. Treatments will be referred to hereafter as: C (control), E10, E20, E40, L10, L20, L40, EL10, EL20, EL40.

All plants were harvested 120 days after treatments were started. It was initially determined whether each plant was alive or dead. Height was measured to the nearest 0.5 cm above the soil surface. Aboveground biomass was separated into leaves and stems, and soil was removed from roots by gently rinsing with water. Plant tissue was dried at 80°C for 48 hr prior to determining leaf weight, stem weight, and root weight. Dry weight/wet weight and aboveground/belowground ratios were used to determine the initial (pre-treatment) biomass distribution of each individual.

Differences among treatments within a species were determined with analysis of variance and Duncan's multiple range test at the $P \leq .05$ level of significance. Differences between species within treatments were determined with a *t*-test at the $P < .05$ level of significance.

RESULTS AND DISCUSSION

Morphological Characteristics and Survival Patterns

Leaf necrosis was observed in most silver maple individuals after 10 days in treatments with early flooding. Leaf senescence was extensive after 20 days, and nearly all leaves had senesced after 40 days. Treatments with only late flooding had considerably less leaf necrosis and senescence. Most individuals had hypertrophied lenticels after 10 days of flooding.

American sycamore had less severe symptons of flooding damage. Extensive necrosis was observed only in treatments with 2-40 days of early flooding, and senescence was prominent only for treatments flooded for 40 days. Most individuals had hypertrophied lenticels after 20 days of flooding. Both silver maple and sycamore resumed leaf growth within a few days after 20 or less days of flooding.

Timing of flooding had a strong influence on the survival of the tree seedlings. Only treatments with early periods of flooding had any mortality. Only 2 out of 8 silver maple and 4 out of 8 sycamore survived the E40 treatment (Table 1). Silver maple had substantial mortality in both EL20 and EL40 treatments, while

sycamore had mortality only in the EL40 treatment. These data suggest that the 1-year old sycamore seedlings had a greater capacity for survival than silver maple following long duration of flooding for the treatments used in this study. Late flooding had no effect on the survival of either species, probably because growth and assimilation processes were greatly reduced by the time the late treatments were initiated.

Growth Parameters

Treatments with early flooding were comparable to those with both early and late flooding for most growth measurements. Treatments with only late flooding generally has less growth reduction compared to the control group for both silver maple and sycamore.

Leaf biomass of silver maple was significantly less than the control group in all E and EL treatments (Fig. 2). Patterns of leaf production were similar between these groups, and there was only a minimal amount of leaf production in E40 and EL40. There was no significant reduction in leaf biomass in the L treatments however.

The actively growing seedlings probably suffered stomatal damage in the early flooding treatments. Stomatal closure is one of the first physiological responses of tree seedlings to flooding (Regehr, et. al., 1975; Sena Gomes and Kozlowski, 1980), and even flood-adapted species have a limited capacity for recovery of stomatal function after inundation. It has been shown that photosynthetic and transpiration rates of silver maple seedlings do not fully recover after 20 days of inundation (Peterson and Bazzaz, in press). The relatively high leaf biomass in the L treatments indicates that late flooding had little effect on leaf growth. This is because plant growth was relatively low when the late flooding period was initiated. Flooding during periods of negligible plant growth generally affects roots more than aboveground production (Teskey and Hinckley, 1977).

Sycamore leaf biomass was significantly reduced only in treatments E40 and EL40 (Fig. 3), which again indicates that early flooding treatments were more damaging. Sycamore leaf biomass was significantly greater than that of silver maple in all E and EL treatments, although control groups were not different. Sycamore appeared to be able to maintain leaf growth better than silver maple following long periods of inundation.

Silver maple stem weight was significantly reduced in all E and EL treatments, but was not reduced in any L treatments (Fig. 2). Sycamore stem biomass was significantly reduced only in treatments E40, L40, and EL40, and there was no difference among these treatments (Fig.3). Sycamore stem weight was greater than that of silver maple in all E and EL treatments, which suggests that sycamore stem growth was less affected by early flooding. Silver maple stem biomass was greater in treatments L20 and L40, however, which suggests that late flooding was detrimental to sycamore stem production. Stem growth of sycamore seedlings may continue later in the season than silver maple.

Silver maple root biomass was significantly reduced in treatments E20, E40, L40, and all EL treatments (Fig. 2). There was negligible root growth in treatments E40 and EL40. Sycamore root weight was significantly reduced in all treatments with 20 or more days of flooding, and there was no significant difference between and 20- and 40-day treatments. Silver maple root biomass was greater in all L treatments, while sycamore root biomass was greater in treatments E40 and EL40.

Early flooding was especially damaging to silver maple root growth, while late flooding was more damaging to sycamore. Flooding has been shown to cause root injury in seedlings of several species (Hosner and Leaf, 1968), and root death and inhibition of root elongation has been observed in previous studies of flooded sycamore seedlings (Tang and Kozlowski, 1982). Reduced root production slows the postflood recovery of seedlings because uptake and transpiration are greatly reduced.

The ratio "root biomass/aboveground biomass" varied considerably between the two species (Figs. 2 and 3). Since this ratio was higher in silver maple than sycamore for control plants, there was probably an inherent difference in biomass distribution for the seedlings. This may account for the higher root weight/aboveground weight ratio in silver maple in most of the treatments. This ratio was significantly reduced for both species in all treatments with 20 or more days of flooding. The root weight/aboveground weight ratio did not decrease beyond 20 days of flooding for either species.

Total biomass of silver maple was significantly reduced in all treatments with early flooding, with negligible growth in treatments E40 and EL40 (Fig. 2). Total weight of sycamore was significantly reduced in all treatments with 20 or more days of flooding (Fig. 3). Silver maple total weight was greater in treatments L20 and L40, while sycamore total weight was greater in all treatments with an early flood of 20 days or more. The susceptibility of silver maple to early flooding and of sycamore to late flooding is again apparent.

In addition to biomass production, height growth is critical for seedlings to compete with understory species for sunlight. This is especially true in energy plantation situations in which tillage practices can encourage annual weed production. Silver maple height growth was significantly reduced in treatments E20, E40, and all EL treatments (Fig. 2). Sycamore height growth was reduced only in treatments with 40 days of early flooding. Silver maple and sycamore had similar height growth across all treatments, so neither species would appear to have a competitive advantage for this growth parameter.

Biomass Increment

In addition to the growth parameters discussed above, the ratio "final weight/initial weight," or biomass increment, is critical in evaluating the relative performance of tree seedlings under different flooding treatments. This parameter provides additional information about the response of species to physiological stress, and indicates the capacity for seedlings to increase their biomass over the course of a growing season.

Silver maple had significant reductions in aboveground biomass increment only in early flooding treatments of 20 days or more (Fig. 4). Sycamore had exactly the same pattern (Fig. 5), and there was no significant differences between species for any of the treatments. Both species were able to substantially increase their aboveground biomass except when subjected to an early flood of 40 days.

Silver maple belowground biomass increment was significantly reduced in all treatments with 20 or more days of flooding (Fig. 4). Again sycamore had the same pattern (Fig. 5). The ratio was greater for silver maple in most cases, including the control, which suggests that greater relative root production was an inherent characteristic of this species.

The total biomass increment for both species was significantly reduced for early flood treatments of 20 days or more (Figs. 4 and 5). Large decrements of

growth were observed as early flood duration increased. In contrast, late flood treatments had no significant effect on biomass increment. Although Figs. 4 and 5 suggest that silver maple total biomass increment was slightly larger than for sycamore, there are no significant differences between species due to large standard errors.

Most silver maple and sycamore seedlings were capable of increasing their aboveground biomass $\geq$ 10 times if they were not subjected to early floods of 20 days or more. Late floods had little effect. Several silver maple treatments achieved nearly a tenfold increase in root production as well, while sycamore had a maximum belowground increment of about 5 times. Late floods did have a measurable effect on root production in both species.

CONCLUSIONS

In this study, we have attempted to differentiate between two species with respect to their suitability for biomass production in flooded environments. A variety of growth parmeters was measured, and conclusions about the relative growth of each species may vary depending upon which parameters are perceived to be most critical in the selection of species.

Aboveground biomass production is a critical factor in seedling growth because this is the portion of the trees which is normally harvested. Early periods of flooding were more damaging to leaf and stem growth in silver maple than in sycamore. However, the aboveground biomass increment for the two species was similar for most treatments, which suggests that they have approximately the same capacity for aboveground production with respect to initial weight. Only sycamore was affected somewhat by late flooding.

Although root biomass is not normally harvested, it plays a role in the physiological vigor of seedlings and indicates how production is allocated. Although seedling root production was inherently greater in silver maple, belowground weight was less than or equal to that of sycamore after 20 days of early flooding. Sycamore root production was affected considerably by late flooding as well as early flooding. The belowground biomass increment of silver maple was higher than that of sycamore in most cases, so although silver maple had lower biomass for some treatments, it had a greater capacity for increasing root production above its initial weight. Since biomass alone does not provide information on the physiological condition of roots, it is difficult to determine which species would have a competitive advantage.

There is no conclusive evidence for selecting either silver maple or American sycamore 1-year old nursery stock as superior for use in biomass plantations which are periodically flooded. Although silver maple may be more sensitive to early flooding, sycamore is more sensitive to late flooding. Overall physiological response, especially as indicated by biomass increment, is quite similar in most cases. Both species have the capacity to recover from moderate levels of flooding and produce substantial quantities of biomass in the first year following planting.

ACKNOWLEDGMENTS

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Table 1. Number of living individuals (out of 8) in each treatment at the end of the 120-day treatment period.

	Treatment									
Species	C	E10	E20	E40	L10	L20	L40	EL10	EL20	EL40
Silver maple	8	8	8	2	8	8	8	8	5	1
American sycamore	8	8	8	4	8	8	8	8	8	6

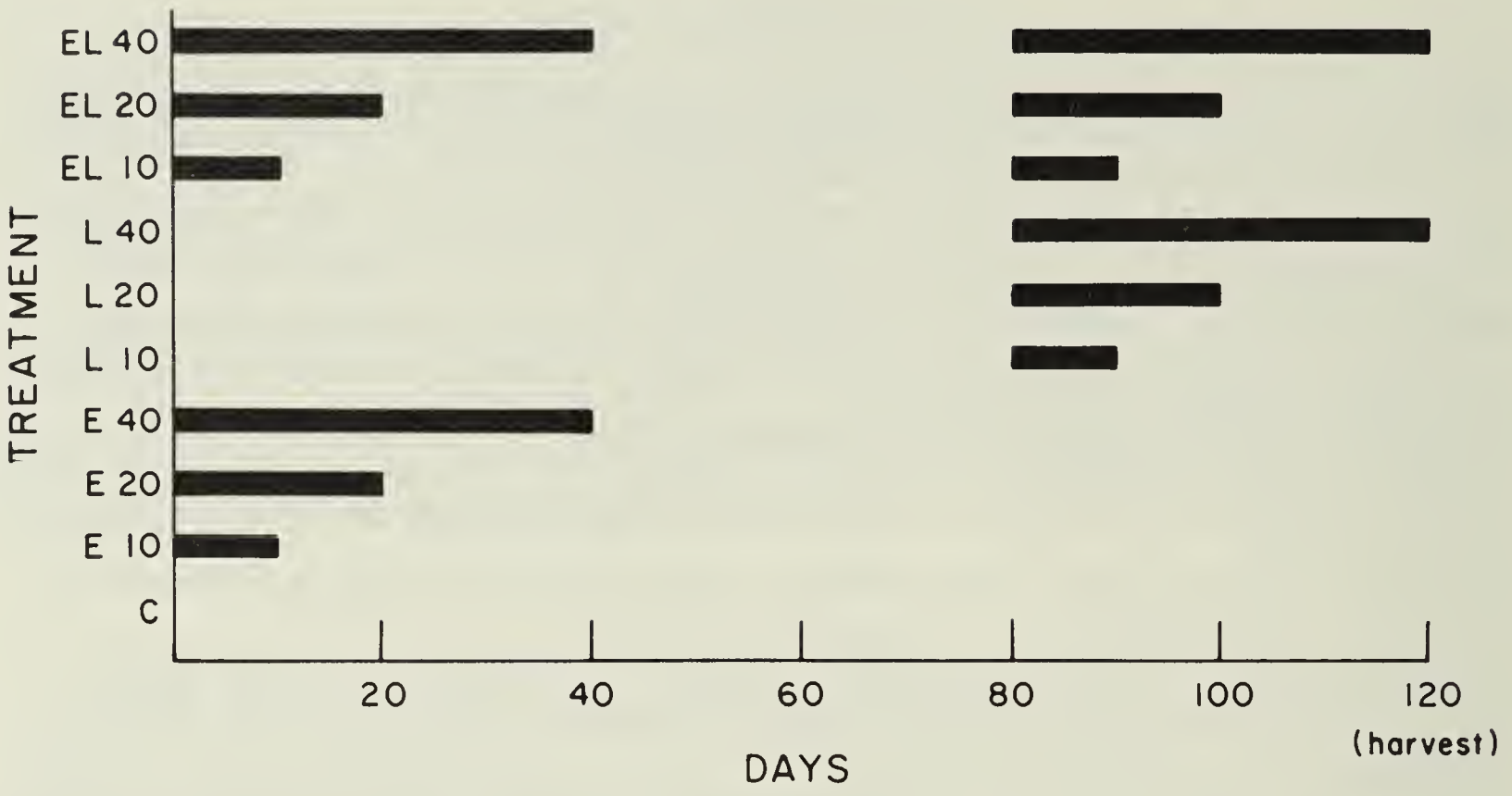

Fig. 1. Treatments used in the study, showing the timing and duration of periods of inundation. Treatments are abbreviated as: C (control), E (early), L (late), EL (early + late). Periods of flooding are 10, 20, and 40 days.

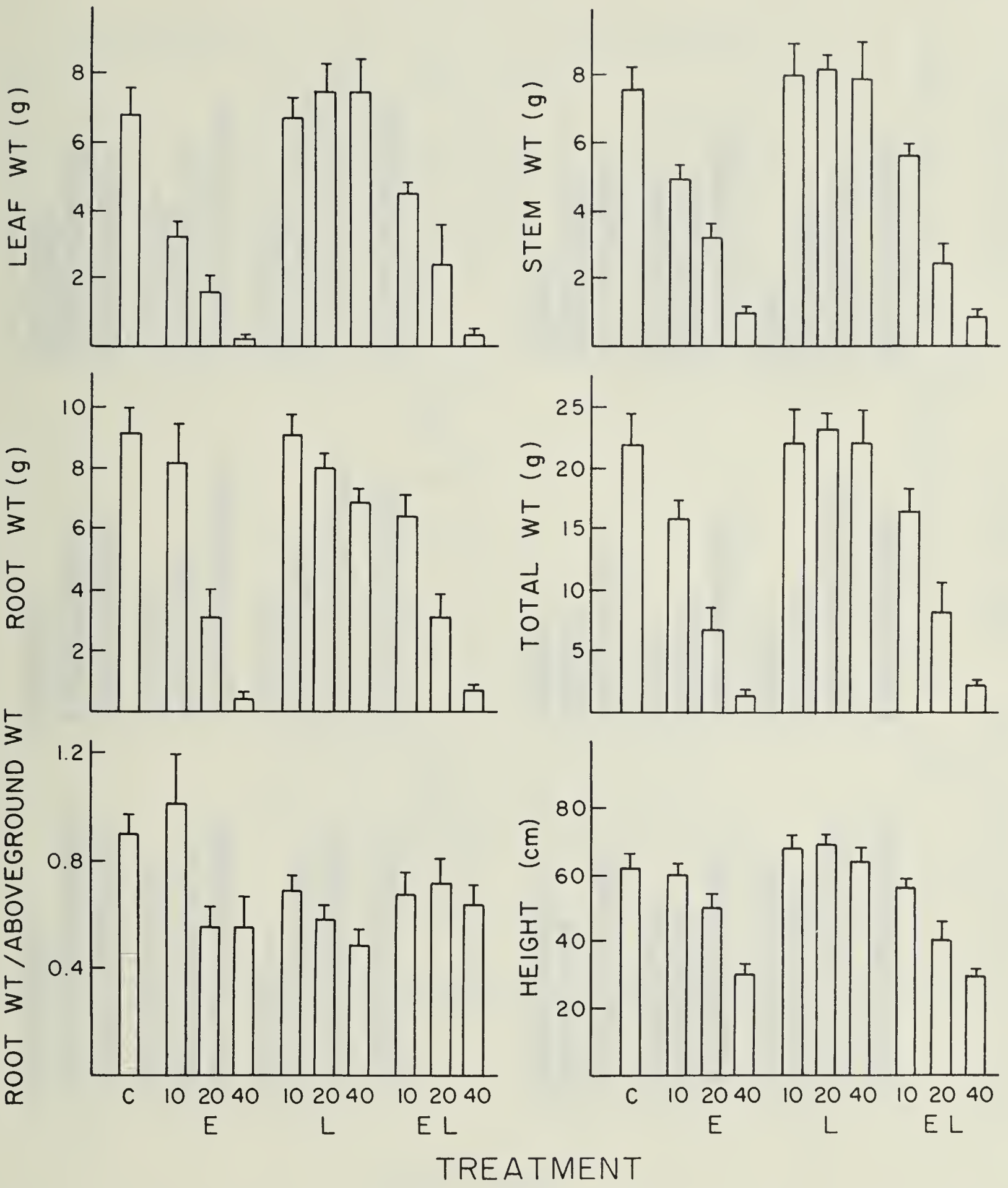

Fig. 2. Growth parameters for silver maple treatments. Aboveground weight is leaf weight plus stem weight. One standard error of the mean is indicated.

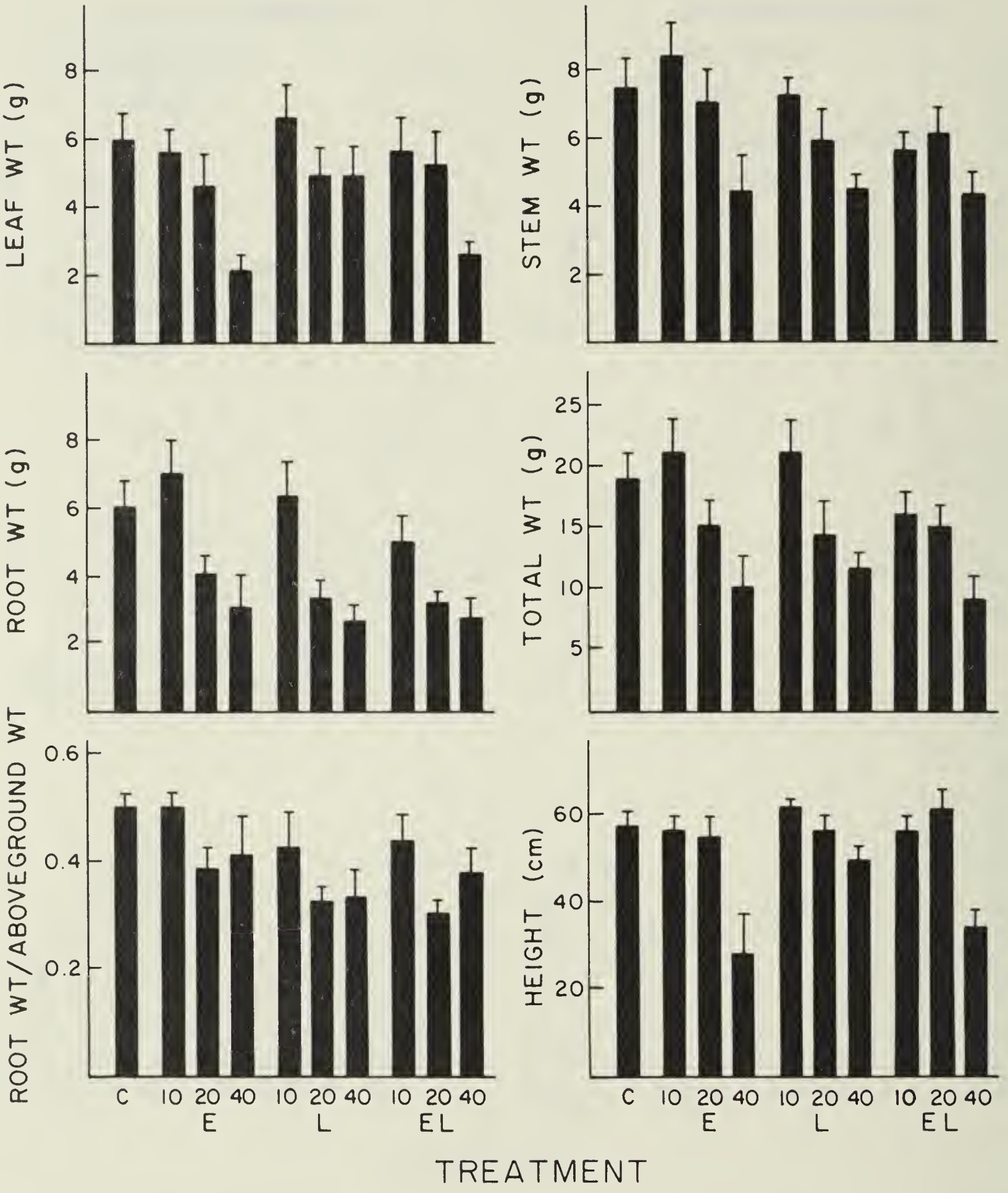

Fig. 3. Growth parameters for sycamore treatments. Aboveground weight is leaf weight plus stem weight. One standard error of the mean is indicated.

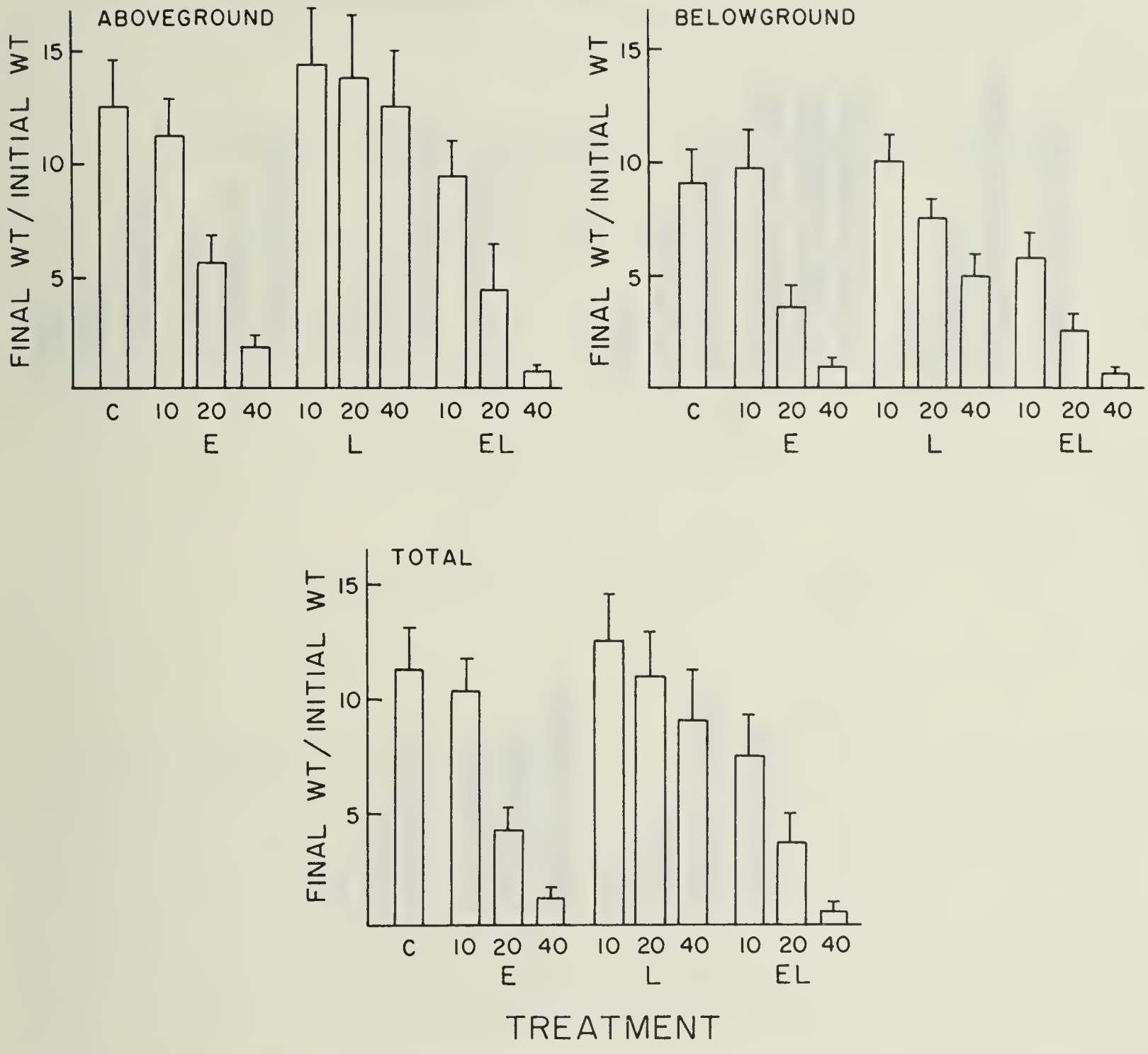

Fig. 4. Biomass increment for silver maple treatments. Aboveground weight is leaf weight plus stem weight. One standard error of the mean is indicated.

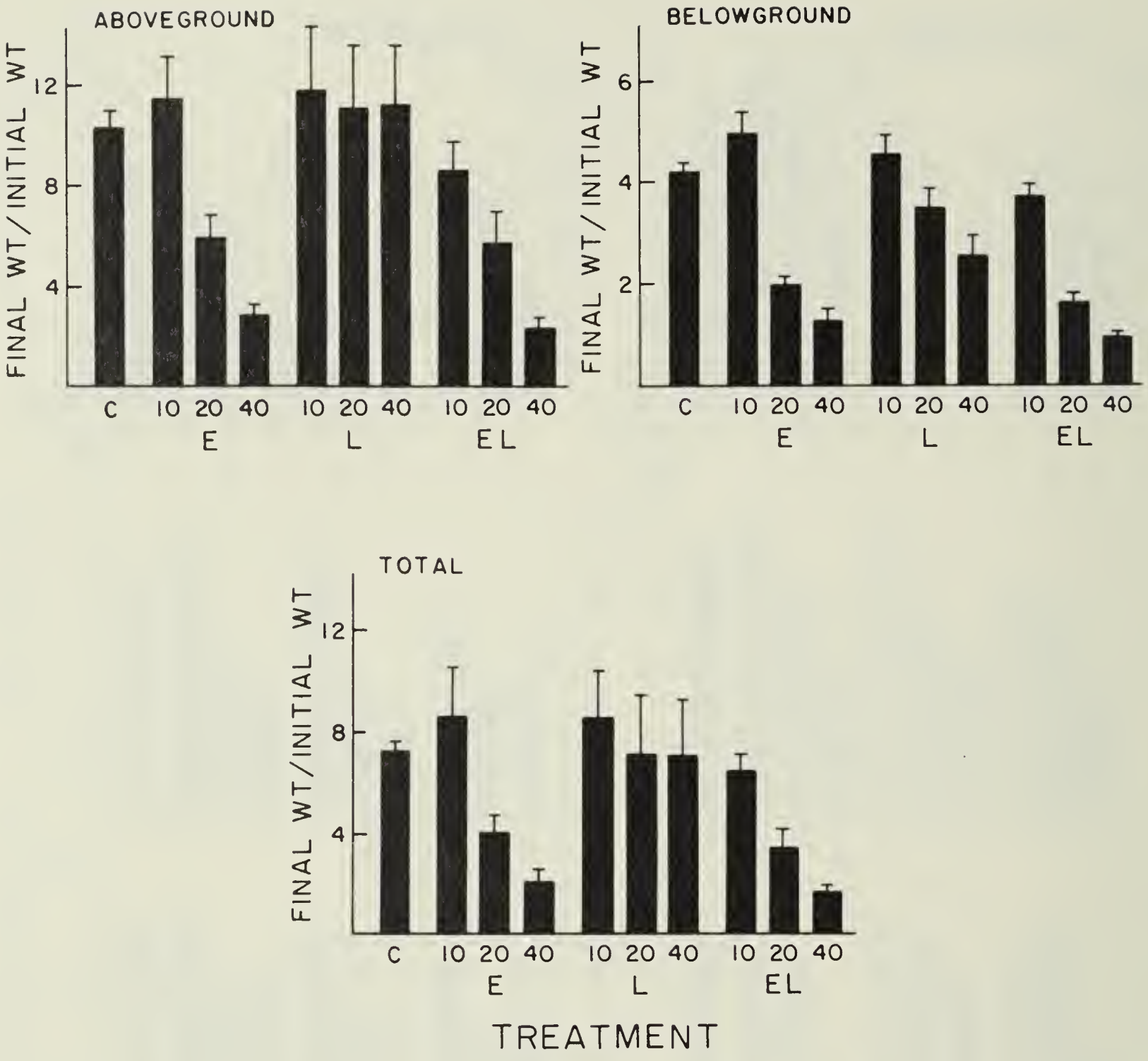

Fig. 5. Biomass increment for sycamore treatments. Aboveground weight is leaf weight plus stem weight. One standard error of the mean is indicated.

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A New Chromosome Configuration in *Drosophila simulans*

By Steven J. Zullo
Department of Biology
University of Notre Dame
Notre Dame, IN
and
Department of Zoology
Southern Illinois University at Carbondale, IL

Drosophila simulans was described by A.H. Sturtevant (1919); when a rsearcher in his laboratory at Columbia University obtained confusing results with field-collected *Drosophila* from Alabama. Males of the Alabama strain mated to mutant *D. melanogaster* females produced only sterile female progeny. The Alabama strain ultimately was identified as *D. simulans*. Until that time, *D. simulans* was not distinguished from *D. melanogaster*, which it very closely resembles. The most reliable differentiating character is the difference in the posterior process of the male genital tergite. The process is clamshell-like in *D. simulans* and is hook-like in *D. melanogaster*.

The *D. melanogaster-D. simulans* cross was the first interspecific hybrid discovered in *Drosophila*. Sturtevant, capitalizing on this discovery and the well-known genetic background of *D. melanogaster*, through a series of experiments determined that any hybrids produced carried a *D. simulans*-X chromosome. That is, in the reciprocal cross between *D. simulans* females and *D. melanogaster* males only males were produced. However some regular females were realized infrequently in this cross. With an attached-X *D. melanogaster* female and a *D. simulans* male patroclinous sons were produced.

Since *D. simulans* was described by A.H. Sturtevant, there have been only two larvae which possessed heterozygous banding sequences of their polytene salivary chromosomes (Dobzhansky 1939, Sturtevant 1931). A female *D. simulans* collected on a Niles, Michigan tomato farm in September 1979 has given rise to a strain which has a salivary chromosome configuration different from that of a *D. simulans* strain from a national stock center. The X and third chromosome banding patterns of the Niles strain *D. simulans* are most distinctive from the laboratory strain *D. simulans*, and from Oregon-R strain *D. melanogaster*.

MATERIALS AND METHODS

All strains were kept on standard cornmeal-molasses-yeast medium at 25°C by mass dump transfer. (The medium at SIU-C was cornmeal-karyo syrup-yeast). Larvae of experimental crosses for cytological study were kept on the same medium at 18°C.

Reciprocal crosses were made between the Niles strain (NILES) and laboratory strain *D. simulans* (LAB). Reciprocal crosses were also made between *D. malanogaster* (ORE-R) and each of the *D. simulans* strains.

Polytene chromosome squashes were made by dissecting the larvae in 45% acetic acid, and immediately transferring the salivary glands to a drop of 1% lacto-acetic orcein stain on a silicon-treated coverslip for 2 minutes. A clean slide was then placed on the coverslip, inverted, and heavy thumb pressure applied.

Chromosomes were examined at 320x with a Zeiss WL phase contrast microscope. The best preparations were recorded on Kodak Panatomic-X, 35mm black and white film.

RESULTS AND DISCUSSION

Crosses between the *D. simulans* strains produced both male and female progeny regardless of the female parents' origin (Table 1), while the crosses between ORE-R and the *D. simulans* strains resulted in characteristic unisexual broods of the *D. melanogaster* - *D. simulans* interspecific cross (Table 2). The males that were produced in the third ORE-R × NILES cross of 1981 were assumed to be XO males, due to non-disjunctional events in the development of the eggs (Sturtevant 1920). The female produced in the only successful LAB × ORE-R cross was one of the last flies produced, and had degenerate ovaries. It was probably due to non-disjunction in the egg and subsequent fertilization of the XX egg by a Y-carrying sperm. At least 45 preparations were made of female salivary gland chromosomes of each strain and experimental cross; at least 6 preparations from each strain and cross were photographed. The quality of the preparations from larvae with *D. melanogaster* female parent was consistently superior to those preparations with *D. simulans* strain female parent, irrespective of the acetic acid batch, lacto-acetic orecin batch, or variation in staining time.

The X-chromosome of the NILES *D. simulans* exhibits a radically different banding pattern than both the LAB *D. simulans* and *D. melanogaster*. There appears to be a band or two missing at the end of the NILES X-chromosome, and possibly a deletion between bands 3A and 3C (Fig. 1). The *D. simulans* NILES strain and LAB X-chromosomes are synapsed only from 3C to 4E in Fig 1b. This is the only cell in which I found this asynaptic/desynaptic state.

The NILES *D. simulans* has an inversion in 3R, encompassing the region between 84B and 92C, with respect to *D. melanogaster* (Fig. 2). This inversion is known to be present in *D. simulans* salivary gland chromosomes with respect to *D. melanogaster* (Patau 1935). The break-points of this inversion have been variously given as 84B3 and 92C3 (Horton 1939), 84E and 93F (Dubinin et al. 1937; *vide* Lemeunier and Ashburner 1976), and 93F6-7 (Ashburner 1969). I am unable to further delineate this inversion due to the generally poor quality of the preparations. This inversion is not seen in hybrids between the two *D. simulans* strains (Fig. 1).

An inversion is present also in 3L of the NILES *D. simulans* with respect to both *D. melanogaster* and the LAB *D. simulans*. It appears to encompass the

chromosome arm from 74A to a region distad of 66A (Fig. 2). This inversion has not been found in the *D. melanogaster* species subgroup (Lemeunier and Ashburner 1976). Again, the quality of the preparations prevented further delineation of the breakpoints. Examination of the male genitalia (unpublished data) indicate NILES is *D. simulans*. In addition, in reciprocal crosses with the LAB *D. simulans*, fertile F_1 were realized (Table 3). I have no explanation for any significant differences from a 1:1 sex ratio. Observation of the mating behavior also indicates NILES is *D. simulans*. The actions of both NILES males and females are consistent with the description of *D. simulans* mating behavior reported by Spieth (1952).

An interesting extension of this discovery would be to compare the niche width of the NILES strain *D. simulans* with strains already in various laboratories, and with strains from more southern climes. Parsons (1975) has pointed out that *D. simulans* is generally found to outnumber *D. melanogaster* in the southern United States, while *D. melanogaster* is more prevalent in the northern states. *D. simulans* is more restricted in its tolerance to temperature fluctuation than *D. melanogaster* (Hosgood and Parsons 1966). Perhaps the chromosome configuration is related to the occurrence of this strain in the northern section of the *D. simulans* range.

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I wish to extend appreciation to Professor Harvey A. Bender, in whose laboratory this research was initiated at Notre Dame; and to Professor Dwayne C. Englert, in whose laboratory it was completed at Southern Illinois University at Carbondale. Special thanks are due R.C. Woodruff for allowing me to deposit this strain in the Mid-America *Drosophila* Stock Center at Bowling Green State University in Bowling Green, Ohio. I have additional data concerning crosses between this strain and various *D. melanogaster* mutants. It is of a preliminary nature, but I believe it is very interesting. I am willing to share it with anyone that may be interested. Yvonne Nolan graciously typed innumerable corrections and her help is greatly appreciated.

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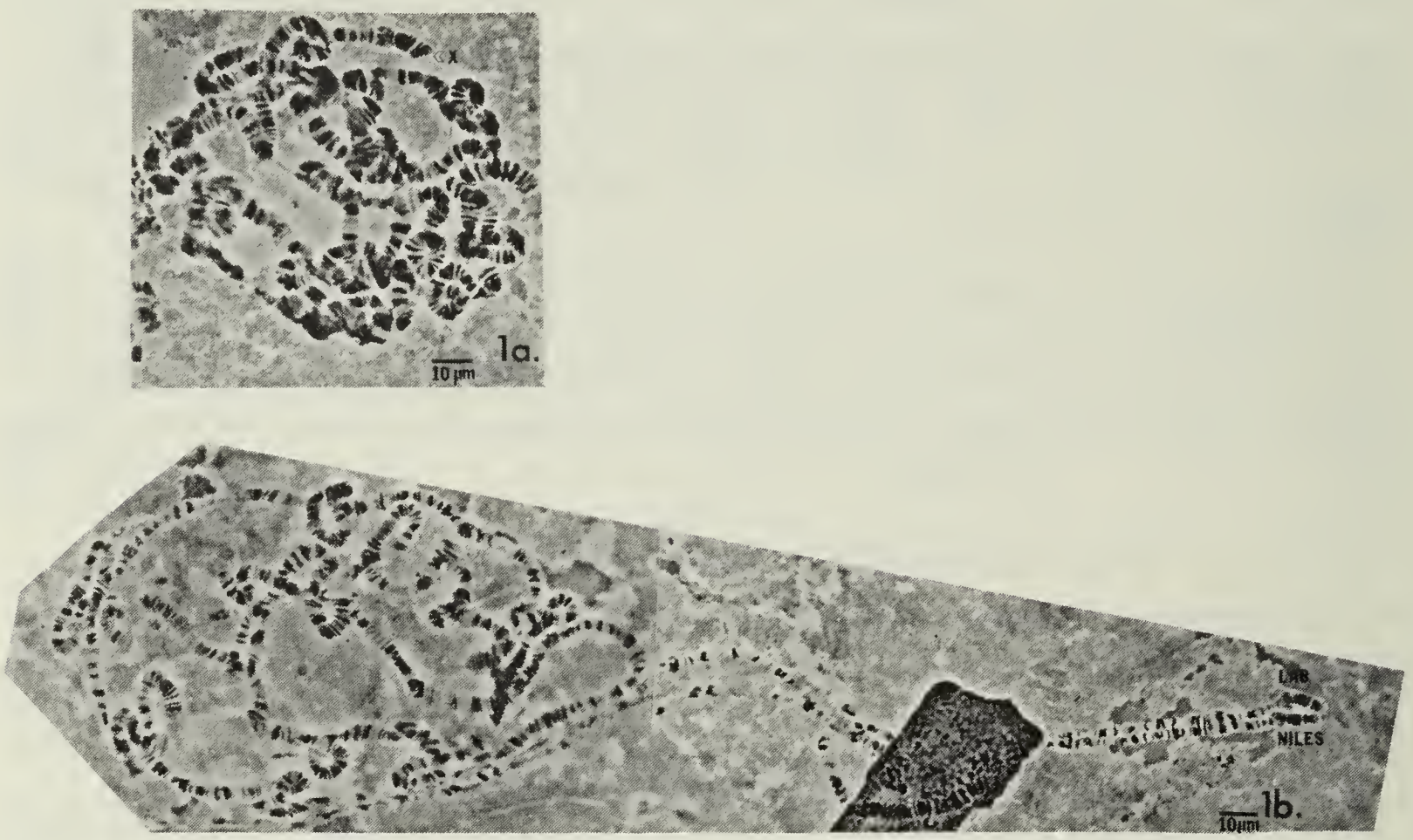

Fig. 1. *Drosophila simulans* hybrid chromosomes.
a) F_1female larva of NILES female × LAB male cross. Arrow indicates shortened X-chromosome of NILES in synapsis with LAB X-chromosome.
b) F_1female larva of different NILES female × LAB male cross than (a) demonstrating different banding patterns of the X-chromosomes of each strain.
Roman numerals indicate chromosome arm (R: right; L: left) Arabic numbers indicate bands.

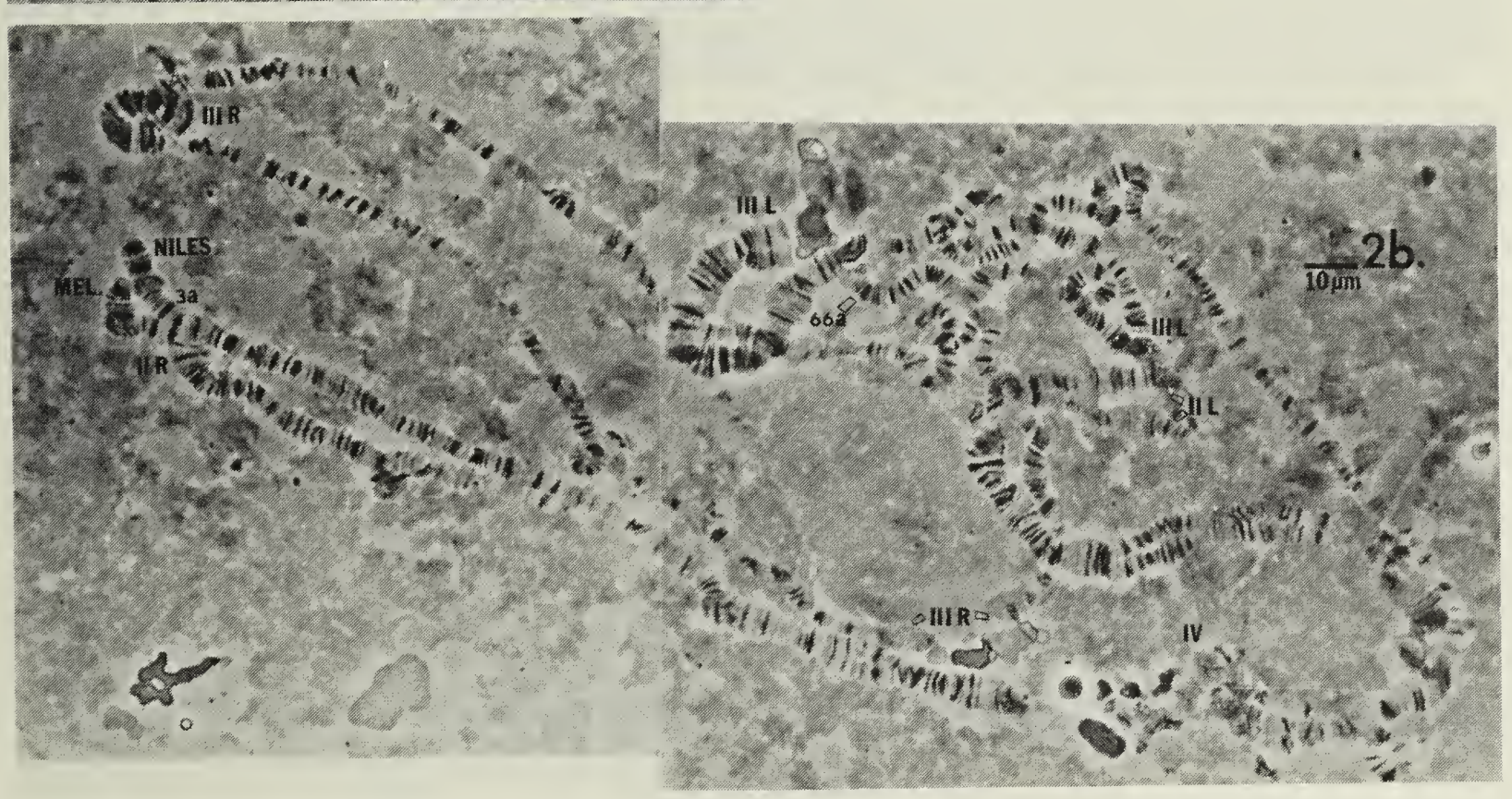

Fig. 2. *Drosophila melangaster-Drosophila simulans* hybrid chromosomes.
a) F_1 female larva of ORE-R female × NILES male cross demonstrating breakpoints of III R inversion.
b) F_1 female larva of different ORE-R female × NILES male cross demonstrating asynapsis in tip of X-chromosomes beyond band 3.
Breakpoint of III L inversion (66a) indicated.
Designations as in Fig. 1.

Table 1. Results of interstrain crosses between *Drosophila simulans* strains from Niles, Michigan (NILES) and laboratory (LAB). Six matings attempted each year, only productive matings listed; Female P_1 listed first. 1981 Medium: Cornmeal-Molasses-Yeast. 1982 Medium: Cornmeal-Karyo Syrup-Yeast.

Cross	Year	Progeny	1	2	3	4	5
LAB × NILES	1981	♀♀	34	27	232	2	
		♂♂	32	27	243	2	
		χ^2	0.06	0	0.25	0	
	1982	♀♀	36	57	26	40	16
		♂♂	29	39	29	33	13
		χ^2	0.75	3.38	0.75	0.67	0.31
NILES × LAB	1981	♀♀	138	145	115	204	133
		♂♂	38	131	80	102	92
		χ^2	0	0.71	6.26*	34.0*	7.47*
	1982	♀♀	95	95	92	94	147
		♂♂	97	95	95	104	117
		χ^2	0.09	0	0.05	0.51	3.41

*$p < 0.05$ ($\chi^2 > 3.84$)

Table 2. Results of interspecific crosses between *Drosophila melanogaster* (OREGON-R Strain: ORE-R) and two strains of *Drosophila simulans* (LAB or NILES). Six matings attempted each year (12 in 1980), only productive matings listed. Female P_1 listed first. 1979-1981 Medium: Cornmeal-Molasses-Yeast. 1982 Medium: Cornmeal-Karyo Syrup-Yeast.

Cross	Year	Progeny	1	2	3	4	5	6	7	8	9
ORE-R × NILES	1979	♀♀	153								
		♂♂	0								
	1980	♀♀	243	455	61	37	2	101	221	70	159
		♂♂	0	0	0	0	0	0	0	0	0
	1981	♀♀	57	67	55						
		♂♂	0	0	2						
	1982	♀♀	53	98	116	62					
		♂♂	0	0	0	0					
NILES × ORE-R	1981	♀♀	0								
		♂♂	28								
	1982		None Successful								
ORE-R × LAB	1981		None Successful								
	1982	♀♀	88	94							
		♂♂	0	0							
LAB × ORE-R	1981	♀♀	1								
		♂♂	111								
	1982		None Successful								

Table 3. Results of interse matings of progeny from 1981 Cross No. 1 and 2 of intrastrain matings (See Table 1).

Parental Cross		F_2 ♀♀	F_2 ♂♂	χ^2
LAB X NILES	No. 1	212	189	1.32
	No. 2	173	164	0.24
NILES X LAB	No. 1	43	37	0.45
	No. 2	208	136	15.07*

*$P < 0.05$ ($\chi^2 > 3.84$)

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Sweet Sorghum Grown on Sludge-Amended Stripmine Soil: A Preliminary Look at Yields, Composition, and Ethanol Production

K.D. Carlson, R.L. Cunningham, and A.I. Herman
Northern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture*
Peoria, Illinois 61604

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

ABSTRACT

Results of a one-year field plot study are reported for sweet sorghum [*Sorghum bicolor* (Moench), variety Wray] grown on stripmine soil in three replications of four soil treatments: control (no amendment); commercial fertilizer; and two levels of sewage sludge amendment (209 and 418 t/ha total dry solids, respectively). Mean sucrose content of whole-stalk dry solids was 35%. Juice yields of 50-68% from fresh stalks were obtained, and the 14% total sugar concentration in the juice was readily fermented by *Saccharomyces cerevisiae* to ethanol in 72% yield. Since sucrose content of the bagasse dry solids was 24%, additional fermentable substrate can be obtained by water extraction. Without additional pretreatment, about 13% of the cellulose in the fresh bagasse was converted to glucose by standard cellulase treatment. More detailed studies are underway on using stripmine land and sewage sludge to produce sweet sorghum as a possible feedstock for fuels and chemicals.

INTRODUCTION

The principal carbohydrates of plant tissues serve as structural components (cellulose and hemicellulose), but large amounts of readily metabolizable carbohydrates (starch and sucrose) may be stored in plants to serve as energy sources (Goldstein, 1981). A major effort has been launched to obtain fermentable sugars from biomass for production of liquid fuels and chemicals. To prepare lignocellulosics

for such conversion, pretreatment processes are being explored that destroy fiber structure and disrupt the lignin-hemicellulose-cellulose complex (Lipinsky, 1979).

Sweet sorghums are varieties of *Sorghum bicolor* (Moench) that accumulate considerable sugar (mainly sucrose) in their stalks during maturation. Although it has been grown for syrup and forage production, sweet sorghum has many attributes as an energy crop based on direct conversion of extracted sugar to ethanol and on energy and chemical production from the associated biomass (Nathan, 1978). With succulent stems, relatively low grain yields, and stalk lengths of 1.8 to 4.3 m, sweet sorghums provide an interesting comparison with commercial hybrid grain sorghums that have drier stems, high grain yields, and attain uniform heights of only 0.9 to 1.2 m (Owen and Moline, 1970). However, as new sweet sorghum varieties are developed for higher sugar yields, grain and biomass yields will become important also in overall economic considerations. Sweet sorghum has appeal as an energy crop because it exhibits great genetic diversity that promises wide adaptability to geographic and climatic conditions, soil types, fertilizer and water requirements, and overall agronomic practices (Nathan, 1978; Coleman, 1970; Jackson and Arthur, 1980; Jackson and Lawhon, 1981).

Recent studies at our Center have shown that crambe and kenaf can be grown on stripmine soil amended with sewage sludge (Carlson *et al.*, 1982). These potential industrial crops — crambe as an oilseed source of erucic acid for production of erucamide (slip and anti-block additive for polyethylene films), and kenaf as a fiber source for newsprint and other types of paper — do not directly involve the food chain. Without diverting current food production acreage, the use of stripmine land and sewage sludge to economically produce sweet sorghum as a feedstock for fuels and chemicals also is conceptually attractive. Both USDA and the Metropolitan Sanitary District of Greater Chicago (MSDGC), cooperator on our most recent work with crambe and kenaf, are interested in energy-from-biomass research programs. In our studies with MSDGC we have observed that sludge amendment of stripmine soils can promote earlier emergence of seedlings from the soil, but that overall germination, emergence, and seedling survival rates may be lower. We have also encountered severe competition by barnyard grass, *Echinochloa crusgalli*, on sludge-treated plots, but we now feel this can be controlled in crambe and kenaf with timely application of postemergence herbicides such as Hoelon®. In practice, seeding rates can be adjusted to give proper plant densities on the amended soils, but grass problems are likely to be more difficult to control chemically with a crop of the Gramineae family (grass) like sweet sorghum.

Our established kenaf plots, which had received annual applications of sludge over a three-year period, provided an immediate opportunity to observe sweet sorghum grown on sludge-treated stripmine soils, thus alerting us to potential problems affecting performance that might be associated with sludge amendment. Pot trials in an environmental growth chamber with sludge-amended soils from the field suggested that soil constituents could affect germination of seeds and development of seedlings. Although preliminary in design, these studies served as valuable indicators of sweet sorghum's likely performance as we planned for more extensive field trials.

We report here the results of our one-year study of sweet sorghum grown in three replications of four treatments: control (C, no amendment); commercial fertilizer (CF); and two levels of sewage sludge amendment (S-209 and S-418). Reported are millable stalk, sugar, and juice yields, whole stalk and bagasse com-

positional data, and evaluations of the expressed juice and bagasse as fermentable substrates.

MATERIALS AND METHODS

Twin sets of experimental plots on stripmine soil on MSDGC property in Fulton County, IL, were first established in 1979 for a three-year study with crambe and kenaf. Each set consisted of 12 plots measuring 15 m × 15 m in a randomized complete block design arranged in a linear array approximately 198 m long and providing 3 replications of 4 soil treatments. In 1981, a 3 m × 198 m strip within and along the south side of the kenaf array provided twelve 3 m × 15 m sorghum plots sharing the soil treatments of the contiguous kenaf plots (east-west rows, 2 m separated sorghum and kenaf).

The plot area was worked to depths in excess of 30 cm in 1979 with equipment designed to pulverize blacktop roadways and rocks. Anaerobically digested sludge (ca. 5% solids) from MSDGC's West-Southwest Sewage Treatment Works was lagooned at the Fulton County site for use in the District's reclamation operations. Sludge solids, later removed from the emptied lagoon, were used in our studies and were 50-69% dry solids when applied by manure spreader to appropriate low- or high-level sludge plots annually at rates of 112 t/ha (S-209) and 224 t/ha (S-418), respectively. Thus, total sludge dry solids applied between 1978 and 1981, and prior to planting the sorghum, were 209 t/ha and 418 t/ha, respectively. Commercial fertilizer, mixed to give 112N/67P/67K kg/ha, was broadcast annually on the appropriate CF plots. Control plots received no amendment except, along with all other plots, an application of potash at the annual rate of 112 kg/ha to counter a generally low K level in the stripmine soil. Annually, prior to seeding and after amendments had been applied, the plots were disced 2 or 3 times crosswise to incorporate sludge and 2 or 3 times lengthwise (long axis of plot area) to further incorporate sludge and plant residues and to prepare the seed bed for planting. Trenches, 0.5 m deep × 1.5 m wide, were cut between plots each year after seeding. Weeds were partly controlled with annual preplant applications of Treflan®at 1.12 kg/ha and by cultivation, but no herbicide was applied to the sorghum strip in 1981.

A single row of sweet sorghum (*S. bicolor*, variety Wray) was planted in the 3 m × 198 m strip on June 1, 1981, with a hand-operated Planet Jr. seeder set to drop seeds at 10-15 cm intervals and a depth of 2.5-5 cm. All plots were cultivated on June 29 to eliminate competing weeds, especially barnyard grass, in sludge-treated plots. Harvest by hand cutting occurred on October 5, 1981, 127 days after planting. Three representative stalks (short, medium, long) were collected from each of the 12 plots, and the replicated samples (9 stalks/treatment, 36 stalks total) were combined to provide composited material for analyses (total representative harvest was 45 kg). Additionally, all stalks were harvested from plots 1-6 (99 m of row), which provided another 378 kg of fresh stalks for a yield estimate and material for storage studies being conducted in another program at the Center. Approximately 60-90 cm of upper stalk and seed head as well as leaves and sheaths were left in the field.

Stalks for expression of juice were sectioned into 5-7.5 cm lengths. Juice was extracted in a slotted, cylindrical, hydraulic press (8.7 cm diam. × 15 cm length) pressurized 3 to 5 times to 1,054 kg/cm^2 (3 or 5 × to 15,000 psig), each time allow-

ing the pressure to drop to 703 kg/cm^2 (10,000 psig). Portions of bagasse from these pressings were repressed similarly to establish maximum juice yields. Juice and bagasse were weighed and immediately frozen until needed.

Chemical composition was determined on sectioned whole stalk or bagasse samples after they were freeze-dried and ground to pass a 1-mm screen in a Wiley mill. Cellulose content was determined by the monoethanolamine (MEA) method (Nelson and Leming, 1957) and reported on an ash- and pentosan-free basis. Lignin analyses were made by the spectrophotometric method (Bagby *et al.*, 1973). Nitrogen was determined by the Kjeldahl method (AOAC, 1980). Water extracts of the freeze-dried and ground whole stalk or bagasse samples were analyzed for soluble sugars by high-performance liquid chromatography (HPLC) with a BioRad (Richmond, CA) HPX-87H size exclusion column and water as the mobile phase. Other analytical procedures were standards of the Technical Association of the Pulp and Paper Industry.

Fermentability of the expressed juice was evaluated as follows: Sterile 60 ml stoppered glass pyrex test tubes containing 17 ml of sorghum juice and 3 ml of Del Rosaria medium (Del Rosario, 1979) were inoculated with 0.1 ml of 24-hr culture of *Saccharomyces cerevisiae* NRRL Y2034. Initial cell counts were 7.0×10^5/ml and the pH was 4.5. Tubes were incubated at 22 C. Over 3 days, 2-ml aliquots were taken at 24-hr intervals for cell counts (haemocytometer) and for determination of glucose/fructose, sucrose, and ethanol concentrations. Sugar analyses were run by HPLC with a 3.9 mm × 30 cm Waters carbohydrate analysis column (Waters Associates, Inc., Milford, MA) and acetonitrile-water (75:25) as mobile phase. Glucose and fructose were not resolved with our procedure. Ethanol concentrations were determined by gas-liquid chromatography.

Thin-layer chromatography (TLC) was performed on commercial precoated plates (0.25 mm silica gel 60 F-254, E. Merck, Darmstadt, Germany). Developing solvent was ethyl acetate:*n*-propanol:methanol:water (55:15:20:7.5) or ethyl acetate:methanol:water (55:40:7.5). Visualization was by charring at 130 C with sulfuric:chromic acid solution or, for colored spot visualization, by heating at 100 C for 10 min after spraying with a fresh solution of ethanol:sulfuric acid:anisaldehyde (9:0.5:0.5) (Waldi, 1965).

Convertibility of cellulose to glucose in sectioned fresh stalks and once-pressed (3 X to 15,000 psig) fresh bagasse was tested by a standard cellulase treatment (Detroy *et al.*, 1980). Thus, 2 to 3.6 g of sectioned fresh stalk material (mean dry solids 28.3%) or 0.9 to 2.7 g of freshly pressed bagasse (49.3% mean yield, 39.3% mean dry solids), representing each of the four soil treatments, were treated for 18 hours with cellulase in a buffered working volume of 20 ml. Comparable buffered samples treated identically, but without cellulase, served as controls. Glucose and sucrose concentrations, for calculating cellulose conversion, were determined on filtrate aliquots by HPLC.

RESULTS AND DISCUSSION

Sweet sorghum yield data are shown in Table 1. Stalk lenghts and diameters ranged from 2.4 to 3.7 m and 2.5 to 5 cm, respectively, with good upright stature. The shorter stalks generally resulted from intense grass competition prior to cultivation, and were mainly in the high-level sludge plots. With an average of 3.42 stalks/m of row and mean weights of 1.25 kg/stalk, each meter of row produced

4.27 kg of fresh stalk after leaves, seed heads, and 60-90 cm of upper stalk had been removed. Total harvest of millable stalks from 99 m of row was 423 kg, which extrapolates to ca. 56 t/ha. Differences due to soil treatments for all parameters in Table 1 are small, with relative standard deviations (RSD) from the means of less than 10%. Yields of juice expressed from fresh stalks with a small roll-type farm mill were comparable to those in the laboratory pressings (ca. 50%) as shown in Table 1. Using the laboratory press, juice yield was increased to 60% by pressing at 5 X to 15,000 psig, and to 68% by re-pressing the bagasse at 5 X to 15,000 psig.

Stalk quarters contributed the following weight percentages to the fresh weight: top quarter, 6%; upper middle quarter, 14%; lower middle quarter, 27%; bottom quarter, 49%; and associated sheaths, 4%. As harvested in this study, the bottom three quarters thus represent 90% of the fresh weight and at least that proportion of juice. Separate pressings of the quarters (at 5 X to 15,000 psig) gave juice yields in the range of 55 to 66% for the three lower quarters, and only 52% for the top quarter. TLC analyses of the separate juice fractions showed no gross differences in the juice sugars, largely sucrose with small quantities of glucose/fructose (not separated). After repressing the combined recovered bagasse, more glucose/fructose was apparent in this juice, suggesting that sucrase action may occur sometime after stalks are initially crushed.

We tested the feasibility of converting cellulose in the fresh sorghum samples by incubating them with a standard cellulase preparation followed by glucose analyses on the resulting reaction media. We found that estimates of cellulose conversion on sectioned fresh stalks were extremely variable and too unreliable for further consideration [mean 5.83% (RSD 242%)]. In contrast, Table 2 shows that cellulase action on the several fresh bagasse samples was very uniform, with a mean cellulose conversion of 13.2% (RSD 6.85%). Cellulosic conversions of 10 to 20% are reasonable for plant material receiving no more pretreatment or size reduction than the bagasse received. To estimate the extent of conversion, glucose initially present in the substrates was subtracted from the glucose levels after cellulase treatment. Furthermore, sucrose levels decreased relative to the buffered controls as a result of cellulase treatment. We assumed that sucrase activity was responsible, and we corrected glucose concentrations accordingly on the basis of 1 g sucrose yields 1.053 g of glucose/fructose (not separately determined). Our conversion estimates in Table 2 will be minimum values if some other process that doesn't generate glucose is responsible for the sucrose disappearance, e.g., a fermentation process. However, we would expect such a process to diminish sucrose levels equally in both the buffered controls and the cellulase-treated samples, and therefore, a differential decrease in sucrose concentration would not have been apparent. Our experience suggests that in using standard cellulase preparations and procedures, it may be necessary to consider that glucose can be generated from substrates other than cellulose, sucrose in our case, and that cellulose conversions could be erroneously high if corrections are not made in these instances.

Fermentability of the sweet sorghum juices was tested in standard incubations with *S. cerevisiae*. Table 3 shows the initial sugar concentrations of juices pressed at 3 X to 15,000 psig, 72-hour fermentation ethanol yields, and the maximum cell counts attained after 48 hours of fermentation. Ethanol yields ranged from 67 to 75% of theory with a mean of 72% (RSD 5.27%) based on initial total sugar concentrations. Mean ethanol concentrations in the fermented juices reached 5.5%

(RSD 4.63%) after 72 hr. We have no information to account for the sugar not converted to ethanol.

Cell populations doubled 6 to 8 times during the fermentations, reaching maxima at 48 hours and declining thereafter (Table 3; Fig. 1). A difference of one generation of cells can account for the apparent population differences by treatment (C S-418 CF~S-209).

Utilization of sucrose and production of ethanol during fermentation are shown as functions of time in Fig. 2. Apparent "induction periods" for sucrose utilization and slightly higher ethanol yields are associated with the control and S-418 juices. These same juices are associated with what may be higher cell populations at 48 or 72 hours (Fig. 1). Since all juices contained similar concentrations of glucose/fructose, which initially would have been used preferentially by the yeast, "induction periods" for sucrose utilization might have been expected for all four juices. However, since these experiments were not replicated, the significance, if any, of the apparent differences in cell populations, sucrose utilization rates, and ethanol yields is not clear at this time.

Compositional data for freeze-dried sectioned whole stalks and once-pressed bagasse are shown in Table 4. The mean sucrose contents (water extractable) of these materials were 35.4% (RSD 18.4%) and 24.3% (RSD 8.2%), respectively. From these data and dry solids contents, fresh stalks as harvested contained at least 9.5-10% sucrose, of which at least 63% is removed in the first-press juice (% juice yield X % sucrose).

Total nitrogen contents of stalks grown on the amended soils are about double the nitrogen content of stalks grown on the control plots. After juice extraction, the nitrogen levels of bagasse from all treatments are similar, suggesting that a soluble form of nitrogen is removed with the juice and is responsible for the higher levels in whole stalks from the fertilized plots.

Alcohol-benzene solubles in the freeze-dried materials ranged from 15 to 35%, or about 4 to 10% on a whole fresh stalk basis, and measures waxes, lipids, and other organics extracted by the hot azeotrope (95% ethanol/benzene, 1:2 v/v). The high sugar content and early stage of maturity may be contributing factors in these relatively high extract levels. Ash levels appear to be somewhat higher in stalks from amended plots as a result of added minerals from the fertilizer and sludge.

Cellulose by monoethanolamine (MEA) extraction ranged from 17 to 20% of the freeze-dried whole stalks and 23 to 26% of the corresponding bagasse. Alpha cellulose values were ca. 1 to 2% lower. Lignin contents of the stalk and bagasse tend to parallel the cellulose and pentosan contents. The pentosan levels in the sorghum bagasse were similar (20-23%), as were the whole stalk levels (15-16%) with the exception of stalks from the CF plots. With the recent finding that the yeast *Pachysolen tannophilus* is capable of direct fermentation of xylose to ethanol (Schneider *et al.*, 1981; Slininger *et al.*, 1982; Detroy *et al.*, 1982a, 1982b), the amount of pentosans (and thus xylose) present in sorghum bagasse is of some significance. In general, pentosans are hydrolyzed under milder reaction conditions than used for cellulose hydrolysis and the remaining residue then is more susceptible to cellulose hydrolysis by enzymes or acid (Lee *et al.*, 1978). Theoretically, xylose from pentosans could be fermented to ethanol by *P. tannophilus*, and glucose, hydrolyzed from the cellulosic residue, could be fermented to ethanol by

S. uvarum (Detroy *et al.*, 1982a) to add to the ethanol obtained from sucrose fermentation.

In summary, with the exception of the need for vigorous grass control measures on sludge-treated soils, we encountered no obvious problems in growing sweet sorghum on stripmine soils. No other negative factors associated with sludge treatment were apparent. Although preliminary, our study serves as a useful indicator of sweet sorghum's performance when grown under these conditions, and the results have proved useful in designing more complete experiments. The data reported should be interpreted within the context of limitations in experimental design and one-year term of the study. Juice yields of 50-68% from fresh stalks were obtained, and the 14% total sugar in the juice was readily fermented by *S. cerevisiae* to ethanol in 72% yield. Since sucrose content of the bagasse dry solids averaged 24%, additional fermentable substrate should be available by water extraction. Moreover, without additional pretreatment, about 13% of the cellulose in the fresh bagasse was converted to glucose by standard cellulase treatment. Approximately 20% and 25%, respectively, of the bagasse dry solids are pentosans and cellulose, which if hydrolyzed would provide additional fermentable substrates.

Based on our results, Fig. 3 provides an estimate of the quantity of ethanol theoretically produced by fermentation of readily extracted sucrose in the sweet sorghum grown in our experiment. Nearly 500 gal (1862 L/ha) is indicated by direct fermentation of the sugar in the expressed juice, and another 300 gal (1200 L/ha) is estimated from fermentation of sucrose that can be washed from the bagasse. Alternatively, sucrose washed from whole stalk dry solids would yield nearly 700 gal/ha (2550 L/ha). All these estimates are based on 72% fermentation yield of ethanol as observed in the direct juice fermentation.

ACKNOWLEDGMENTS

We thank C.L. Joseph, W.L. Orton, D.M. Palmer, and M.I. Schulte for their technical assistance. Sorghum seed was supplied by K.C. Freeman and D. Broadhead of the U.S. Sugar Crops Field Station, U.S. Department of Agriculture, Meridian, MS. The support and cooperation of G. Hall, R.I. Pietz, and L. Klindworth of the Metropolitan Sanitary District of Greater Chicago is appreciated.

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Table 1. Yields of sweet sorghum grown on stripmine soils.

Soil treatment	Fresh stalks*		Juice†	Bagasse†	
	Yield, kg	Dry solids, %	Yield, %	Yield, %	Dry solids, %
Control (C) (No Amendment)	10.9	28.1	51.1	48.9	37.6
Comm. fertilizer (CF) (112N/67P/67K, kg/ha)	12.8	27.9	53.1	46.9	38.6
Sewage sludge (S-209) (209 t/ha dry solids)	11.0	27.7	48.4	51.6	40.5
Sewage sludge (S-418) (418 t/ha dry solids)	10.3	29.5	50.2	49.8	40.3
Mean	11.2	28.3	50.7	49.3	39.3
(RSD)‡	(9.52)	(2.97)	(3.85)	(3.96)	(3.55)

*Yield is total weight of 9 stalks, 3 harvested from each replicate to form the treatment composite.

†Single pressing of fresh stalks at 3 X to 15,000 psig.

‡Relative standard deviation, %.

Table 2. Cellulase treatment of sweet sorghum bagasse.*

Soil treatment	Concentrations, g-%/g substrate: Sucrose, Net Decrease†	Glucose: Gross Increase‡	Glucose: Sucrose Equivalent§	Glucose: Net Increase¶	Cellulose Conversion,# %
Control (C) (No Amendment)	- 0.38	0.59	0.40	0.19	14.6
Comm. fertilizer (CF) (112N/67P/67K, kg/ha)	- 0.40	0.58	0.42	0.16	12.5
Sewage sludge (S-209) (209 t/ha dry solids)	- 0.12	0.31	0.13	0.18	12.5
Sewage sludge (S-418) (418 t/ha dry solids)	- 0.61	0.81	0.64	0.17	12.6
Mean	- 0.38	0.57	0.40	0.18	13.2
(RSD)	(52.9)	(35.6)	(52.9)	(6.11)	(6.85)

*Mean bagasse yield from fresh whole stalks was 49.3% (RSD 3.96%), and the mean dry solids was 39.3% (RSD 3.55%). Cellulase treatment of fresh bagasse (0.92-2.24 g wet, or 0.37-0.86 g dry solids) according to Detroy et al. (16) with buffered controls. RSD = relative standard deviation, %.

$$\#\,\%\ \text{conversion} = \frac{\text{Net glucose X 0.9 X 20}}{\text{MEA cellulose (\%)}}\ \text{X 100.}$$

†Sucrose levels decreased with cellulase treatment, relative to buffered controls.

‡Difference between cellulase treated samples and buffered control samples.

§Calculated quantity of glucose and fructose (not separated) assumed to be formed from sucrose that disappears during the reaction. Glucose + fructose (MW = 360) /sucrose (MW = 342) = 1.053 X decrease in sucrose concentration.

¶Column 3 minus column 4.

Table 3. Fermentation of sweet sorghum juice from plants grown on stripmine soil.*

Soil treatment	Initial concentration of juice sugars: Glucose-Fructose,† %	Sucrose, %	Total, %	Concentration of ethanol produced: Final, %	% of Theory‡	Maximum cell count, 48 hour X 10^{-5}/ml
Control (C) (No Amendment)	2.23	11.8	14.0	5.63	74.7	1500
Comm. fertilizer (CF) (112N/67P/67K, kg/ha)	2.05	12.7	14.7	5.30	67.0	700
Sewage sludge (S-209) (209 t/ha dry solids)	1.83	12.4	14.3	5.37	69.9	800
Sewage sludge (S-418) (418 t/ha dry solids)	2.07	12.5	14.6	5.86	74.6	800
Means	2.05	12.4	14.4	5.54	71.6	950
(RSD)§	(8.04)	(3.12)	(2.20)	(4.63)	(5.27)	(38.9)

*Juices from a single pressing at 3 X to 15,000 psig, mean yield from fresh stalks = 50.7% (RSD 3.85%).

†Not separated by analytical technique, assumed to be a mixture.

‡Based on total initial sugar concentration.

§Relative standard deviation, %.

Table 4. Chemical composition of sweet sorghum grown on stripmine soil.*

Soil treatment	Chemical composition†							
	Solubility in Alcohol-Benzene, %	Cellulose by MEA, %	Cellulose Alpha %	Pentosans %	Lignin %	Ash %	Nitrogen %	Sucrose %
Control (C) (No Amendment)	25.1 (30.4)	17.0 (23.5)	15.6 (21.4)	15.2 (22.7)	16.1 (9.0)	2.57 (2.52)	0.26 (0.42)	37.5 (24.5)
Commerical fertilizer (CF) (112N/67P/67K, kg/ha)	25.2 (29.2)	16.7 (23.1)	16.0 (21.7)	8.9 (20.1)	16.7 (10.9)	3.26 (2.74)	0.65 (0.55)	43.2 (26.9)
Sewage sludge (S-209) (209 t/ha dry solids)	35.0 (24.2)	17.8 (25.9)	16.9 (24.1)	16.2 (20.6)	12.2 (11.3)	2.63 (2.68)	0.50 (0.39)	28.0 (24.0)
Sewage sludge (S-418) (418 t/ha dry solids)	15.2 (20.0)	19.9 (24.3)	18.5 (22.7)	16.3 (20.4)	19.9 (19.2)	2.68 (2.64)	0.56 (0.53)	32.8 (22.0)

*Analyses on freeze-dried material ground to pass 1 mm screen. Values in ( ) are for bagasse.

†Mean dry solids of whole fresh stalks and bagasse were 28.3% (RSD 2.97%) and 39.3% (RSD 3.55%), respectively. Mean bagasse yield was 49.3% (RSD 3.96%). RSD = relative standard deviation, %.

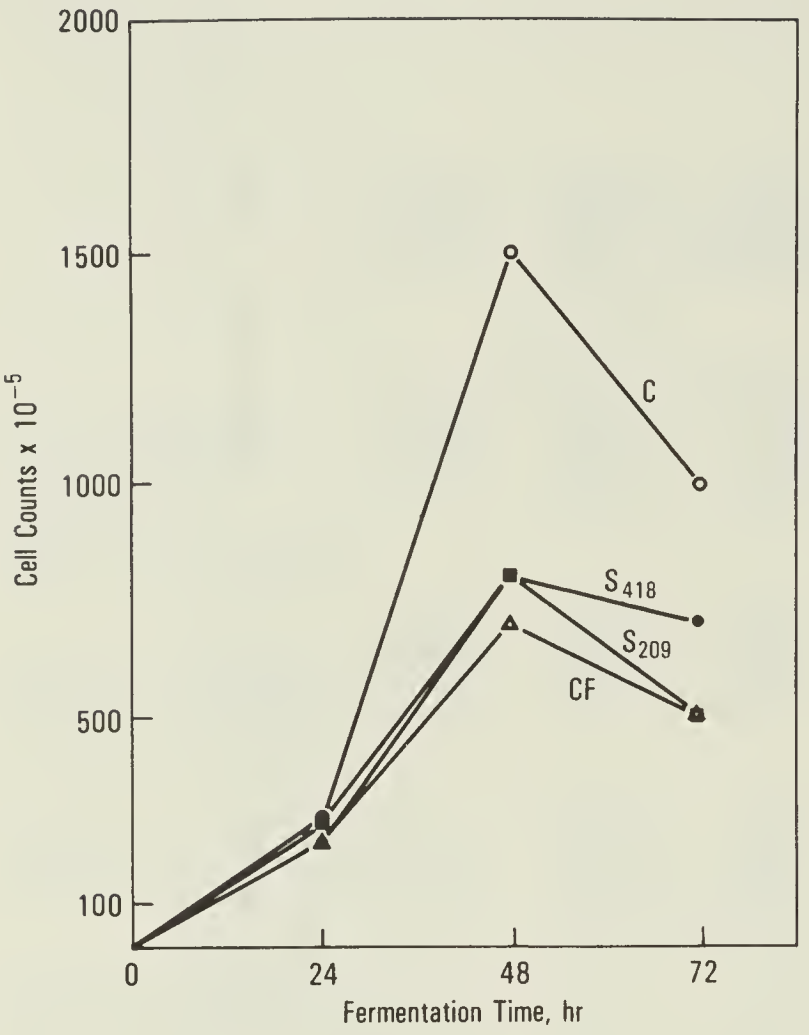

Fig. 1. Fermentation of sweet sorghum juice: Cell counts as a function of time and plot treatment.

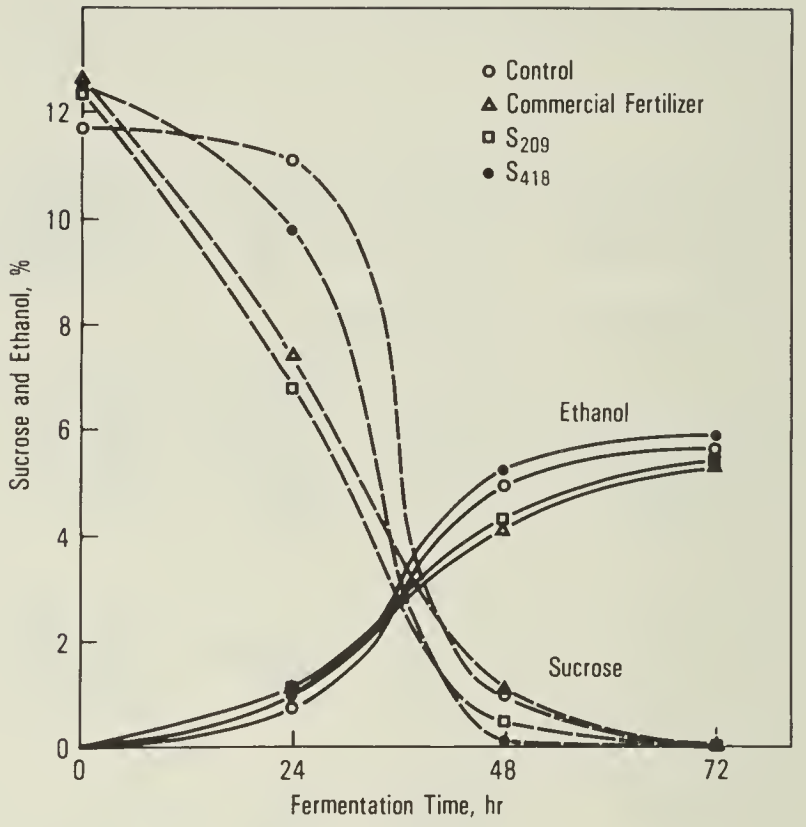

Fig. 2. Fermentation of sweet sorghum juice: sucrose utilization and ethanol production as a function of time and plot treatment.

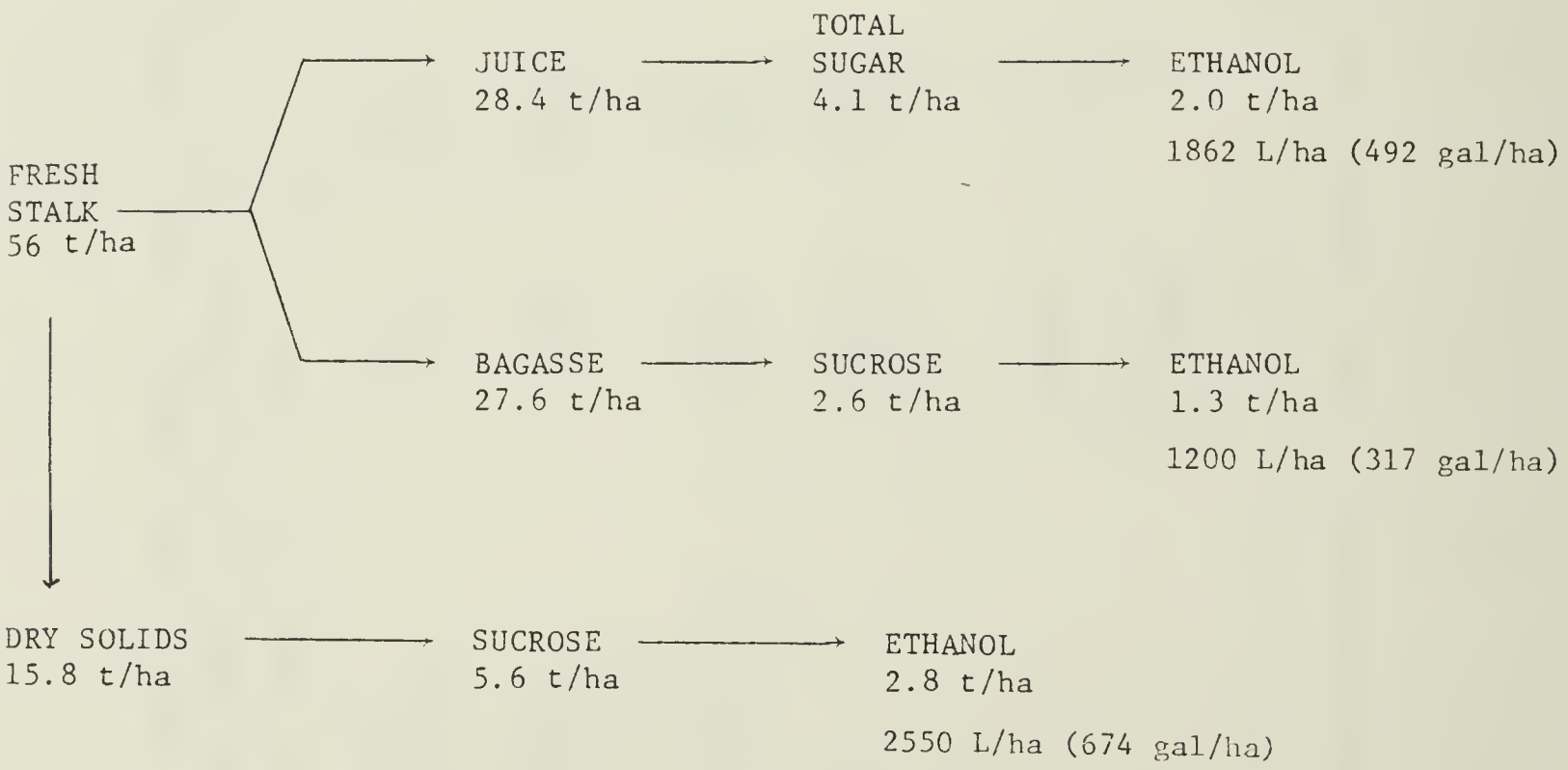

Fig. 3. Theoretical ethanol yields from total juice sugars, and from sucrose obtained from sweet sorghum bagasse or whole stalk solids.

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Study of Tobacco Mosaic Virus Replication In Protoplasts of *Saccharomyces Cerevisiae*

Amrik S. Dhaliwal and Gurdip Kaur
Department of Biology
Loyola University of Chicago
6525 North Sheridan Road
Chicago, Illinois 60626

ABSTRACT

The objective of this research was to study the effect of temperature, time and concentration of Tobacco Mosaic Virus (TMV inoculum) on its replication in isolated *Saccharomyces cerevisiae* (yeast) protoplasts. The protoplasts were prepared by treating the yeast cells with enzyme B — Glucuronidase/Aryl Sulfatase. Suspensions of protoplasts in osmotic stabilizer were infected with a common strain of TMV and incubated at 28°C for five hours. TMV-infected protoplast samples were taken at half-hour intervals, washed three times with osmotic stabilizer, suspended in 0.1 M phosphate buffer pH 7.0 and then TMV replication was assessed by inoculating ten day old primary leaves of *Phaseolus vulgaris* cv. Pinto, and by staining the infected protoplasts with fluorescent antibodies.

Both the assay methods used to determine the extent of TMV replication indicated the maximum increase at four hours of incubation. TMV-RNA replication may have taken place earlier but the whole virus was detected two and one half hours after inoculation. TMV replication in yeast protoplasts seemed to be temperature dependent. Maximum infectivity was obtained when protoplasts infected with TMV were incubated at 28°C. Protoplasts inoculated with 10X virus dilution showed double the infectivity as compared to 100X and 1,000X dilutions.

INTRODUCTION

Viruses are nucleoproteins, highly specific to their hosts. It has been established that animal virus can infect the animal cell through the process of pinocytosis, but such phenomenon is absent in the case of plants because plant cell walls act as a barrier against the entrance of the virus.

Lederberg (1956) suggested that protoplasts are useful for studying the biosynthesis of plant viruses. Takebe, Otsuki, and Aoki (1968) successfully isolated protoplasts from tobacco leaf infected with Tobacco Mosaic Virus (TMV). Using the fluorescent antibody staining technique, Otsuki and Takebe (1969) located TMV antigen in infected tobacco protoplasts. Coutts, Cocking, and Kassanis (1972)

successfully isolated *Saccharomyces cerevisiae* (yeast) protoplasts and infected them with TMV.

The purpose of this research is to isolate protoplasts of *Saccharomyces cerevisiae*, infect them with a common strain of TMV and study the replication process of TMV using fluorescent antibody staining technique and conventional assay procedure using *Phaseolus vulgaris* cultivar Pinto as assay host under different conditions such as temperature, duration of incubation, and concentration of TMV inoculum.

MATERIALS AND METHODS

Cultivation of *Saccharomyces cerevisiae*

A common strain of *Saccharomyces cerevisiae* cultivar ellipsoideus 556, obtained from the American Type Culture Collection, was maintained on agar slants at 4°C. The yeast was grown for cell isolation in 125 ml Erlenmeyer flasks containing 30 ml of the pre-culture liquid medium at 25°C for 24 hours. Inoculum from the 24 hour culture was transferred into another 125 ml Erlenmeyer flask containing 30 ml of the shake culture liquid medium and was grown for 18 hours at 25°C with constant agitation. Yeast cells from the 18 hour culture were collected by centrifugation in an International Clinical Centrifuge at approximately 1000 rpm for 5 minutes. Cells were washed twice with glass distilled water and then used for the isolation of protoplasts. The composition of the agar medium, preculture liquid medium, and shake culture liquid medium is given in Table 1 (Darling *et al.*, 1969).

Isolation of Protoplasts

One gram wet weight of yeast cells was suspended in an osmotic stabilizer (sorbitol-citric acid pH 5.0) and incubated for 30 minutes at 30°C with occasional agitation. Yeast cells were collected by centrifugation and treated with 2 ml of enzyme B — Glucuronidase/Aryl sulfatase from the gut juice of snail (Helix pomatia). The mixture of yeast cells and enzyme was incubated at 38°C with occasional agitation. A maximum number of protoplasts were obtained within 3 to 4 hours. The enzyme and the cell wall debris were removed by centrifuging at approximately 1000 rpm for 5 minutes and the protoplasts were then suspended in fresh osmotic stabilizer.

Inoculation of Protoplasts with TMV

The protoplast suspension in osmotic stabilizer was mixed with an equal volume of the common strain of diluted purified TMV (1:50 in osmotic stabilizer). TMV was purified by using Venecamp and Mosch method (1963). This mixture, used as inoculum, produced an average of 100 lesions per half leaf. 0.5 ml of the mixture was taken in sterile test tubes immediately after mixing TMV with protoplasts and this sample represented the zero time incubation. The protoplasts were washed three times with osmotic stabilizer, and divided into two parts. One part was suspended in 0.25 ml of osmotic stabilizer and slides were prepared for fluorescent antibody staining and microscopy. The other part was suspended in 0.25 ml of phosphate buffer and was kept in a freezer for bioassay. The remaining protoplast-virus mixture was incubated at 28°C. Samples were taken from this mixture at half-hour intervals for the first 5 hours and at longer intervals thereafter. Infected protoplasts were washed three times with osmotic stabilizer and were treated and studied in the same manner as the protoplasts from the zero time sample.

The data were analyzed statistically using log transformation, analysis of variance technique, and t-test for independent sample means.

Assay of Infectivity:

Fluorescent Antibody Staining of Infected Yeast Protoplasts

TMV infected protoplasts of *Sacchraomyces cerevisiae* were stained with 1:8 dilution of the fluorescent antibody stain (Clark and Shepard, 1963) following the method of Otsuki and Takebe (1969) and observed under the fluorescent microscope for stained protoplasts (Fig. 1).

Bioassay

Phaseolus vulgaris cultivar Pinto was used as an assay host. Plants were grown in the greenhouse and 10 day old leaves were inoculated with TMV infected protoplasts according to the method described by Lamborn, Cochran and Chidester (1971).

Lesions were counted with a Wild binocular microscope using 500X magnification. Color and morphology of the lesions were observed in order to find out whether the lesions were caused by intact TMV or TMV-RNA alone. Light brown and spreading type lesions are mostly caused by viral RNA, whereas, TMV produces dark brown and compact lesions.

RESULTS AND DISCUSSION

Effect of Time of Incubation On the Replication of TMV in *Saccharomyces cerevisiae* Photoplasts

The lesions produced by 0.5 hour sample may be due to the adsorption of virus particles on the surface of the yeast protoplasts. The progressive decrease in the number of lesions at 1.0, 1.5, 2.0, and 2.5 hours of incubation might be due to the fact that as virus particles enter the protoplasts through the process of pinocytosis, the protein coat of the virus is removed and the virus enters the eclipse phase, during which time the virus particles are not detected. After the removal of the protein coat, the viral nucleic acid in the protoplasts probably acts as mRNA and combines with the host ribosomes to form polysomes which are responsible for the synthesis of polymerases and other enzymes required for the multiplication of the nucleic acid of the virus. Increase in infectivity at 3.0 hours after inoculation is probably due to the appearance of new copies of the viral RNA which reached maximum value at 4.0 hours. The decrease in the number of virus particles after 4.0 hours could possibly be due to the destruction of viral RNA by RNAase present in the host cell before the RNA is enclosed in the protein coat. The number of infectious particles again increased at 20.5 hours and 47.0 hours, followed by a drop at 120.0 hours. The infectivity was more with the 47.0 hour sample as compared to 4.0 hour samples (Fig. 2). This decrease and increase in infectious particles might be due to the quick synthesis and degradation of the viral RNA before it was enclosed in the coat protein which was synthesized at later stages of the viral replicative cycle. It is well known that viral RNA is degraded by the ribonucleases while RNA enclosed in the coat protein is not affected by the nucleases.

It is clear from this data that virus synthesis continued at least up to 120.0 hours. If virus synthesis was complete, that is, RNA synthesized was enclosed in coat protein, the infectivity would have remained at the level as indicated at 47.0 hours of incubation. The fact that infectivity decreased indicated the degradation of RNA which was recently synthesized and was not enclosed in coat protein.

TMV infected protoplasts stained with fluorescent antibodies stain followed the same pattern of replication as indicated by the bio-assay.

Effect of Temperature on the Replication of TMV in *Saccharomyces cerevisiae* (Yeast) Protoplasts

Saccharomyces cerevisiae protoplasts were inoculated with TMV and incubated at 15°C, 28°C, and 45°C. Samples were taken at half hour intervals from all three temperatures for infectivity tests. Samples taken at 0 time and one-half hour after inoculation showed the infectivity due to the adsorbed virus. The decrease in infectivity up to 2.0 hours incubation probably indicates the period during which viral synthesis occurs in the protoplasts. The trend in increase or decrease in infectivity is the same in the three incubating temperatures under study. Samples taken from 15°C, 28°C, and 45°C showed that the maximum infectivity occurred at 4.5, 7.0, and 8.0 hours respectively, thus indicating that 28°C is the optimum temperature for TMV replication in yeast protoplasts (Fig. 3).

Effect of Inoculum Concentration on the Replication of TMV in *Saccharomyces cerevisiae* Protoplasts

Virus suspensions containing 500 lesion forming units was diluted 10X, 100X, and 1000X. Yeast protoplasts were inoculated with these virus dilutions, and incubated at 28°C for 5.0 hours. Samples of the inoculated protoplasts were taken every half hour and tested on half leaves of *Phaseolus vulgaris* cultivar Pinto, the results of which are shown in Fig. 4. The 0 time and half-hour incubation periods for all three dilutions showed the presence of adsorbed virus particles as indicated by the lesions produced on the assay host. The lesser the inoculum dilution, the more the virus adsorbed on the surface of the protoplasts. However, the adsorption was about the same at the dilution of 100X and 1000X. Infection, using 100X and 1000X dilutions, started decreasing at 1.0 hour and continued until 3.0 hours, which probably indicates the eclipse phase of the viral replicative cycle. Infectivity increased after 3.5 hours of incubation period and decreased again at 4.0, 4.5, and 5.0 hours of incubation. The infectivity in the protoplasts inoculated with 10X dilution increased after 4.0 hours of incubation.

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Table 1. Composition of slant culture medium, preculture medium, shake culture medium and protoplast medium for the maintenance of yeast and yeast protoplasts.

	Grams/Liter			
	Shake Culture Medium	Protoplast Medium	Slant Culture Medium	Preculture Medium
Potassium dihydrogen orthophosphate	0.20	0.20	2.00	2.00
Magnesium sulfate	0.25	0.25	1.00	1.00
Glucose	0.10	1.00	20.00	20.00
Yeast extract	3.00	3.00	3.00	3.00
Sodium lactate	10.00	1.00	—	—
Ammonium hydrogen phosphate	3.50	3.50	—	—
Sorbitol	—	127.52	—	—
Ammonium sulfate	—	—	1.00	1.00
Difco peptone	—	—	3.50	3.50
Difco special agar	—	—	30.00	—

Fig. 1. Saccharomyces cerevisiae protoplasts four hours after inoculation with tobacco mosaic virus at 35°C under 100 ft. c. light intensity. Photographed after staining with fluorescent antibodies. Magnification 1000X.

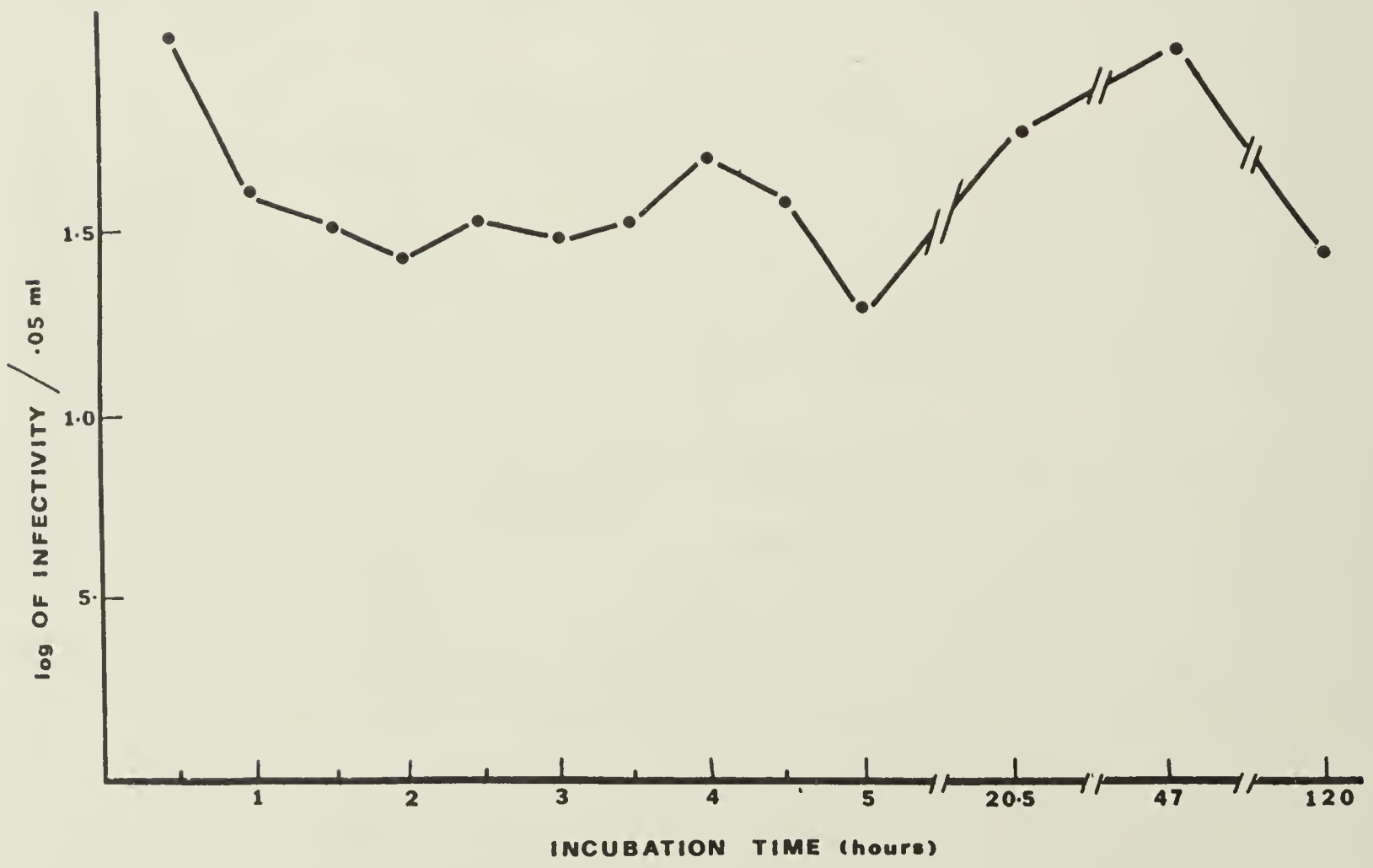

Figure 2. Effect of time of incubation on the replication of tobacco mosaic virus in Saccharomyces cerevisiae protoplasts assessed by local lesion assay on six half leaves of Phaseolus vulgaris cv. pinto.

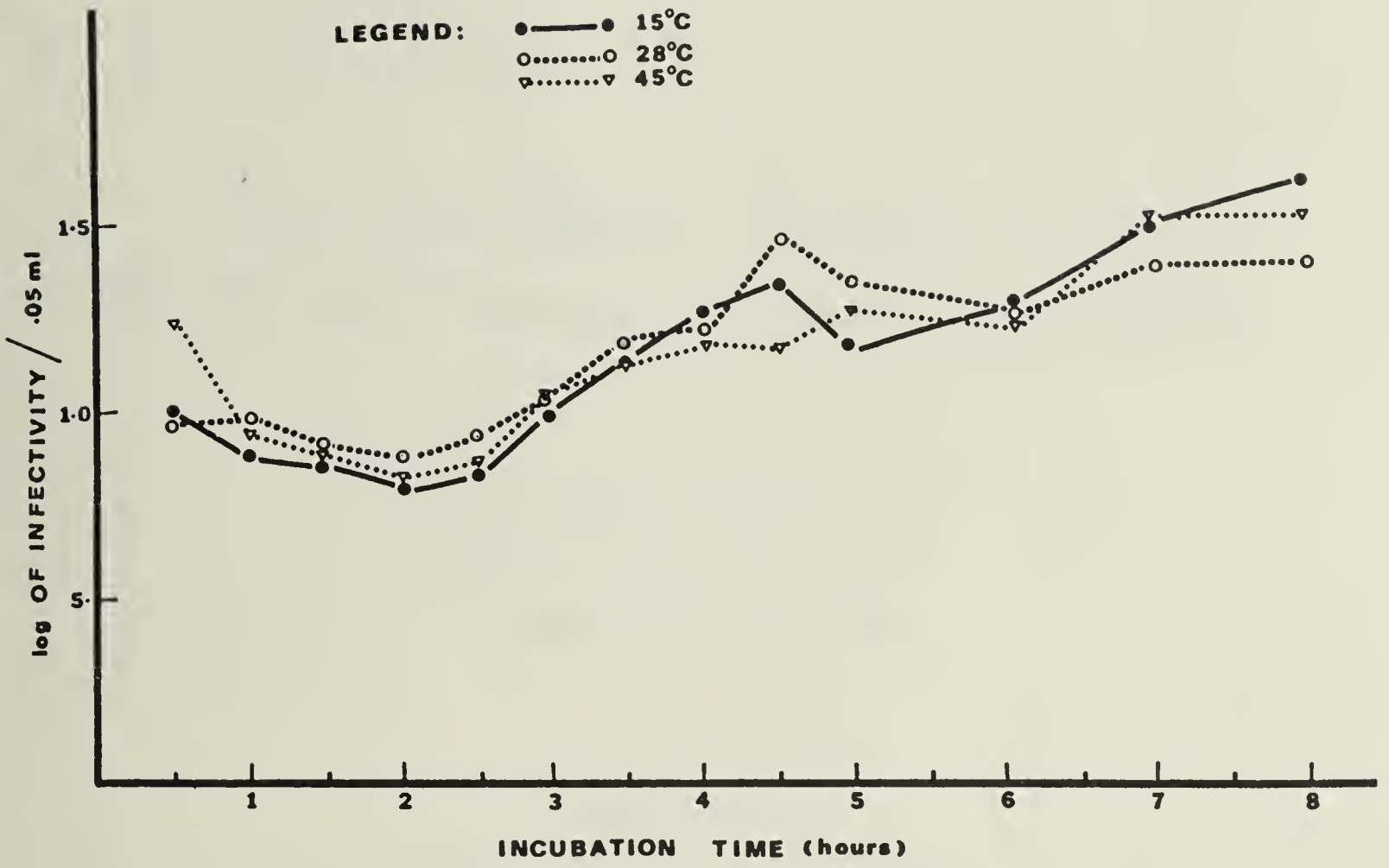

Figure 3. Effect of temperature on the replication of tobacco mosaic virus in Saccharomyces cerevisiae protoplasts assessed by local lesion assay on six half leaves of Phaseolus vulgaris cv. pinto.

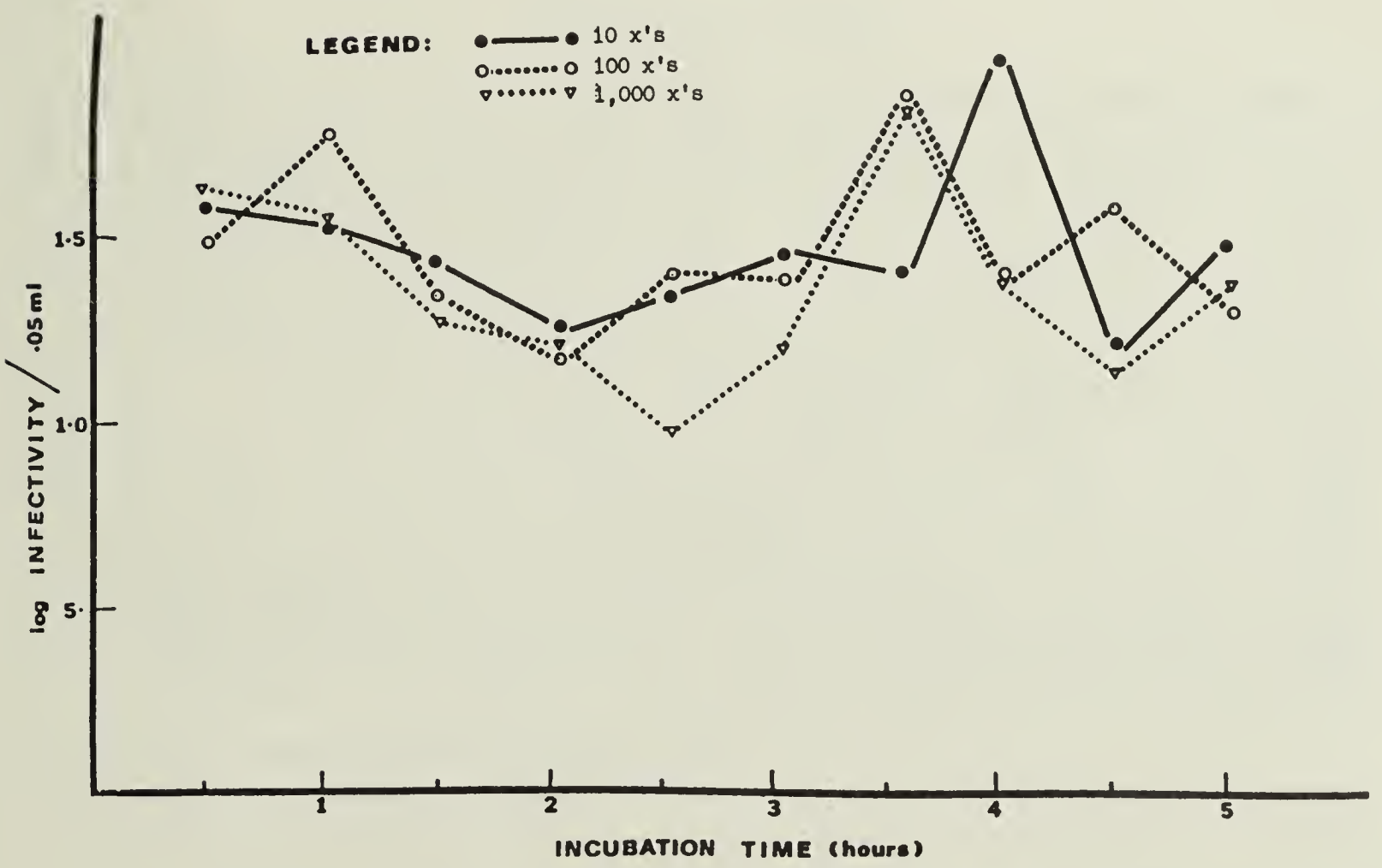

Figure 4. Effect of concentration of virus inoculum on the replication of tobacco mosaic virus in Saccharomyces cerevisiae protoplasts assessed by local lesion assay on six half leaves of Phaseolus vulgaris cv. pinto.

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The Pallid Shiner, *Notropis amnis* Hubbs and Greene, A Rare Illinois Fish

Thomas M. Skelly and Michael J. Sule[1]
Illinois Natural History Survey
607 East Peabody Drive
Champaign, Illinois 61280

ABSTRACT

The pallid shiner, *Notropis amnis*, a minnow thought to have been extirpated from Illinois, was collected in the Kankakee River in Will County, near Custer Park in 1978, 1979, 1981, and 1982. Specimens were collected in lentic areas of the river having negligible current and a sand-silt substrate. Thirty-six other species of fishes were collected in seine hauls with *N. amnis*, the most common being *N. spilopterus*, *Pimephales notatus*, *P. vigilax*, *N. stramineus*, and *Lepomis humilis*. Cluster analyses of the minnows, using water quality variables, indicated that *N. amnis* preferred conditions most similar to those chosen by *P. vigilax* and *P. promelas*.

INTRODUCTION

The pallid shiner, *Notropis amnis* Hubbs and Greene, is one of the rarest and least known American fishes (Smith, 1979). Its range extends from the Mississippi River in Minnesota and Wisconsin south to the Amite River in Louisiana and west to the Guadalupe River in Texas (Clemmer, 1980). This fish is rare in the northern half of its range, and since 1970 most specimens have been taken from backwaters of the Mississippi River in Wisconsin, primarily between the Wisconsin-Illinois border and Prairie du Chien (Don Fago, Wisconsin Department of Natural Resources, personal communication). This species has shown the most marked decline of any fish in Missouri and may have been eliminated from that state (Pfleiger 1975). It was thought to be extirpated from Illinois until its recent collection in the Kankakee River. Since 1978, Illinois Natural History Survey biologists have collected 18 specimens, 15 in August collections and 3 in November. Prior to these collections only 22 specimens of *N. amnis* were considered Illinois records, the last collected in 1963 (Table 1). This note will add to the information on this

[1]Present Address: Illinois Department of Conservation,
P.O. Box 223, Oregon, Illinois 61061

rare species by describing the habitat, water-quality parameters, and species associations at collection locations. Specimens upon which this paper is based are retained in the Ichthyological Collection of the Illinois Natural History Survey, Champaign, Illinois.

SITE DESCRIPTION

Fishes were collected in the Kankakee River, 0.6 km to 1.1 km north of Custer Park, Will County, Illinois, approximately 23 km upstream of its confluence with the Des Plaines River. Pallid shiners were collected at Stations 3R, 5L and 5R within the Braidwood Aquatic Monitoring Area (Figure 1). This area incorporates the riverside intake (3R) and discharge (4R) structures for Commonwealth Edison's Braidwood Generating Station, scheduled for on-line activity in 1985. Station 3R was located on a narrow river reach approximately 125 m wide. When a single specimen of *N. amnis* was collected at Station 3R in 1978, water velocity was negligible there due to low flow, which exposed a riffle immediately upstream that channeled water to the opposite bank. Seining areas at Station 3R were characterized by a steep-sided, shaded, muddy bank with some undercut roots. The substrate was primarily silt-covered bedrock. Stations 5L and 5R were within a wide (250 m) area of the river, lentic in nature. Station 5 was approximately 500 m downstream from Station 3. The shoreline at Stations 5L and 5R was mildly sloping with sparse aquatic vegetation. The substrate at unshaded Station 5L was primarily fine sand and silt with some large rocks and cinder blocks near the bank. The substrate at partially shaded Station 5R was predominantly fine sand with some silt.

METHODS

All fishes were collected using a 7.65-m by 1.22-m nylon seine (King 4.76 mm^2 mesh) that included a 1.22 -m by 1.22-m by 1.22-m bag. Two discrete 15-m sites within each station were seined once on each of two separate dates approximately 1 week apart. These four seine hauls represented the total seining effort at a station during a given sampling month. Sampling was conducted in May, August, and November 1977-79 and in August 1981-82. All small fishes were fixed in 10-percent formalin, returned to the laboratory, soaked in tap water for 24 hours, and preserved in 70-percent ethanol. After preservation, specimens were measured for total length and weight. Large fishes were processed in the field and released.

Water-quality measurements for each station included current velocity, temperature, dissolved oxygen, depth, turbidity, pH, and conductivity. Due to the proximity of the two sampling sites within a station, one set of water-quality values was used to characterize that station on a given day. These characteristics were assigned to each fish collected, and weighted mean parameter values for each species were calculated from fishes collected at all stations during August 1979. Cluster analysis (Statistical Analysis System 1979; Standardized, Mean = 0, Standard Deviation = 1) incorporating these mean values was performed on all minnows (Cyprinidae) to detect natural assemblages of species.

RESULTS AND DISCUSSION

Notropis amnis from the Kankakee River was found in areas of slight current, no measurable current, and backflow (Table 2). Although turbidities during collecting periods were low, the river is subject to dramatic increases in turbidity (as high as 300 NTU) during storm periods. All *N. amnis* were collected in seinable areas at water depths no greater than 1.5 m. Water temperatures varied seasonally, with specimens collected in water 6.0°C to 29.5°C. These quantitative data supplement earlier obsrevations, such as turbidity ranging from "clear" to "very muddy" (Hubbs, 1951) and that *N. amnis* is somewhat tolerant of turbidity (Clay, 1975). Pflieger (1975) states that this species is intolerant of excessive siltation and turbidity. Several studies have shown that *N. amnis* resides in slowly moving waters (Hubbs, 1951; Miller and Robison, 1973; Clay, 1975; Pflieger, 1975). When collected from the Mississippi River, it has been found in flowing water over sand bars (Harlan and Speaker, 1969; Eddy and Underhill, 1974).

Station 5, where most of the specimens were collected (10 at Station 5L, 7 at 5R, 1 at 3R), was a slack-water, depositional area despite fluctuating levels of river discharge — unlike other stations within the study area that were periodically scoured by currents. Year-round slow current and depositional substrate appear to be the preferred habitat of *N. amnis*, since no specimens were collected in any other habitat type despite extensive sampling throughout the monitoring area.

Good water quality throughout the study area, as indicated by the analysis of 54 water quality parameters (Skelly and Sule, 1980), and a variety of habitats are important to the maintenance of the diverse Kankakee River assemblage of fishes, totaling at least 72 species. The Kankakee River in Illinois is noted for its good water quality, diverse aquatic life, and scenic beauty (Ivens et al. 1981).

Notropis amnis seldom enters the mouth of tributary streams (Hubbs, 1951; Eddy and Underhill, 1974). The lentic characteristics within the mouth of Horse Creek, a third order stream (Horton-Strahler system) entering the Kankakee River within the study area, were quite similar to those of Station 5, yet no pallid shiners were collected from that creek. Hubbs (1951) suggested that *N. amnis* in its northern range is confined to the large lowland rivers, possibly because large rivers are warmer than small to medium-sized streams. Small streams like Horse Creek are also subject to sudden increases in current that may be unacceptable to *N. amnis*.

Fishes collected in seine hauls at stations where *N. amnis* was collected included 36 other species representing 8 families (Table 3). Species and abundance of fishes collected varied by date and station. However, the most common associations and those found during each sampling period included *Notropis spilopterus* and *Pimephales notatus*. *P. vigilax* and *Notropis stramineus* were abundant during several collections, and *Lepomis humilis* was occasionally common. *N. spilopterus* is the only member of this group that is considered tolerant of high gradient streams. The others are characteristic of quiet pools and backwaters of medium to moderately large streams. Both *N. spilopterus* and *L. humilis* are somewhat tolerant of turbid conditions (Pflieger, 1975).

The cluster analysis of minnows (Figure 2), based on weighted mean water-quality values assigned to each species (Table 4), showed that *N. amnis* was quite distant from most species except *P. vigilax* and *P. promelas*. *Pimephales vigilax* was restricted to the slowly moving right side of the river and Station 5L during August 1979, whereas *P. notatus* was common throughout the study area, and hence, its distance from *N. amnis* in the cluster.

Notropis amnis has not been officially listed as endangered or threatened in Illinois, because it was believed to have been extirpated (Brigham et al., 1981). This species, now known to exist in Illinois waters, has been proposed by the Illinois Endangered Species Technical Advisory Committee on Fishes for consideration as a threatened species. Additional studies, especially on the Mississippi and Kankakee Rivers, are critical if the exact status of this fish in Illinois is to be determined.

Authors' Note: Additional information concerning *Notropis amnis* is now available in: Becker, G.C. 1983. Fishes of Wisconsin. University of Wisconsin Press, Madison. 1052 pp.

ACKNOWLEDGMENTS

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Table 1. Locations where *Notropis amnis* has been collected in Illinois.

River	Location	County	Date	Number of Specimens	Collectors	Catalogue Number
—	Berwyn	Cook	1900	3	A. P. Ford	FMNH 61753[a]
Rock	Oxbow Island	Rock Island	10 July 1925	3*	—	—
Sangamon	3.2 km W Dewey	Champaign	17 Aug 1928	2	Thompson & Hunt	INHS 87962[b]
Sangamon	2.4 km S Fisher	Champaign	17 Aug 1928	1	Thompson & Hunt	INHS 87960
Sangamon	1.6 km E Fisher	Champaign	18 Aug 1928	2	Thompson & Hunt	INHS 87961
Little Muddy	8.0 km E Tamaroa	Jefferson	Oct 1940	2	A C. Bauman	UMMZ 163053[c]
Mississippi	6.4 km W Blackhawk	Carroll	19 Aug 1963	1	Russell & Bystry	INHS 21843
Kankakee	8.8 km ESE Ritchie	Will	Oct 1963	8	Muench	INHS 4860
Kankakee	1.1 km N Custer Park	Will	9 Aug 1978	1	Sule	INHS 27071
Kankakee	0.6 km N Custer Park	Will	9 Nov 1978	1	Sule	INHS 27078
Kankakee	1.1 km N Custer Park	Will	15 Nov 1978	2	Sule	—
Mississippi	3.2 km N Cordova	Rock Island	12 April 1979	1	Lutterbie	INHS 27091
Kankakee	1.1 km N Custer Park	Will	9 Aug 1979	1	Sule	INHS 27603
Kankakee	1.1 km N Custer Park	Will	15 Aug 1979	8	Sule	INHS 27602
Kankakee	1.1 km N Custer Park	Will	6 Aug 1981	2	Skelly	INHS 87557
Kankakee	1.1 km N Custer Park	Will	11 Aug 1981	1	Skelly	INHS 97559
Kankakee	1.1 km N Custer Park	Will	2 Aug 1982	2	Skelly	INHS 87959

*Specimens not examined. Reported in Hubbs (1951).
[a]Field Museum of Natural History, Chicago, Illinois.
[b]Illinois Natural History Survey, Champaign, Illinois.
[c]University of Michigan Museum of Zoology, Ann Arbor, Michigan.

Table 2. Physical measurements and water quality parameters associated with specimens of *Notropis amnis* collected from the Kankakee River, Illinois, in 1978, 1979, 1981, and 1982.

Date	Station	Specimen	TL mm	Wt. g	KTL	Vel. Cm/Sec	pH	Turbidity N.T.U.	Depth m	Temp °C	D.O mg/l	Conductivity μmhos/cm
9 Aug 1978	5R	1	41	0.42	0.61	4.0	9.2	15	1.5	27.2	10.0	625
9 Nov 1978	3R	1	45	0.74	0.81	0.0	8.6	3	0.5	11.0	11.8	600
15 Nov 1978	5L	1	54	1.12	0.71	0.0	8.7	7	0.5	6.0	13.8	500
15 Nov 1978	5L	2	53	1.13	0.76	0.0	8.7	7	0.5	6.0	13.8	500
9 Aug 1979	5L	1	38	0.41	0.75	1.2	8.8	37	0.5	27.6	7.2	690
15 Aug 1979	5L	1	40	0.52	0.81	0.0	8.2	27	0.5	21.9	7.4	600
15 Aug 1979	5L	2	40	0.47	0.73	0.0	8.2	27	0.5	21.9	7.4	600
15 Aug 1979	5L	3	43	0.59	0.74	0.0	8.2	27	0.5	21.9	7.4	600
15 Aug 1979	5R	1	40	0.40	0.62	-2.8	8.2	37	0.5	20.4	7.3	600
15 Aug 1979	5R	2	41	0.42	0.61	-2.8	8.2	37	0.5	20.4	7.3	600
15 Aug 1979	5R	3	39	0.39	0.66	-2.8	8.2	37	0.5	20.4	7.3	600
15 Aug 1979	5R	4	41	0.48	0.70	-2.8	8.2	37	0.5	20.4	7.3	600
15 Aug 1979	5R	5	28	0.13	0.59	-2.8	8.2	37	0.5	20.4	7.3	600
6 Aug 1981	5L	1	36	0.33	0.71	-5.2	7.9	32	0.6	23.0	6.9	580
6 Aug 1981	5L	2	31	0.23	0.77	-5.2	7.9	32	0.6	23.0	6.9	580
11 Aug 1981	5R	1	22	0.16	1.50	0.0	8.0	50	0.6	23.0	7.2	520
2 Aug 1982	5L	1	38	0.37	0.67	0.0	8.4	16	0.5	29.5	9.6	660
2 Aug 1982	5L	2	33	0.25	0.70	0.0	8.4	16	0.5	29.5	9.6	660

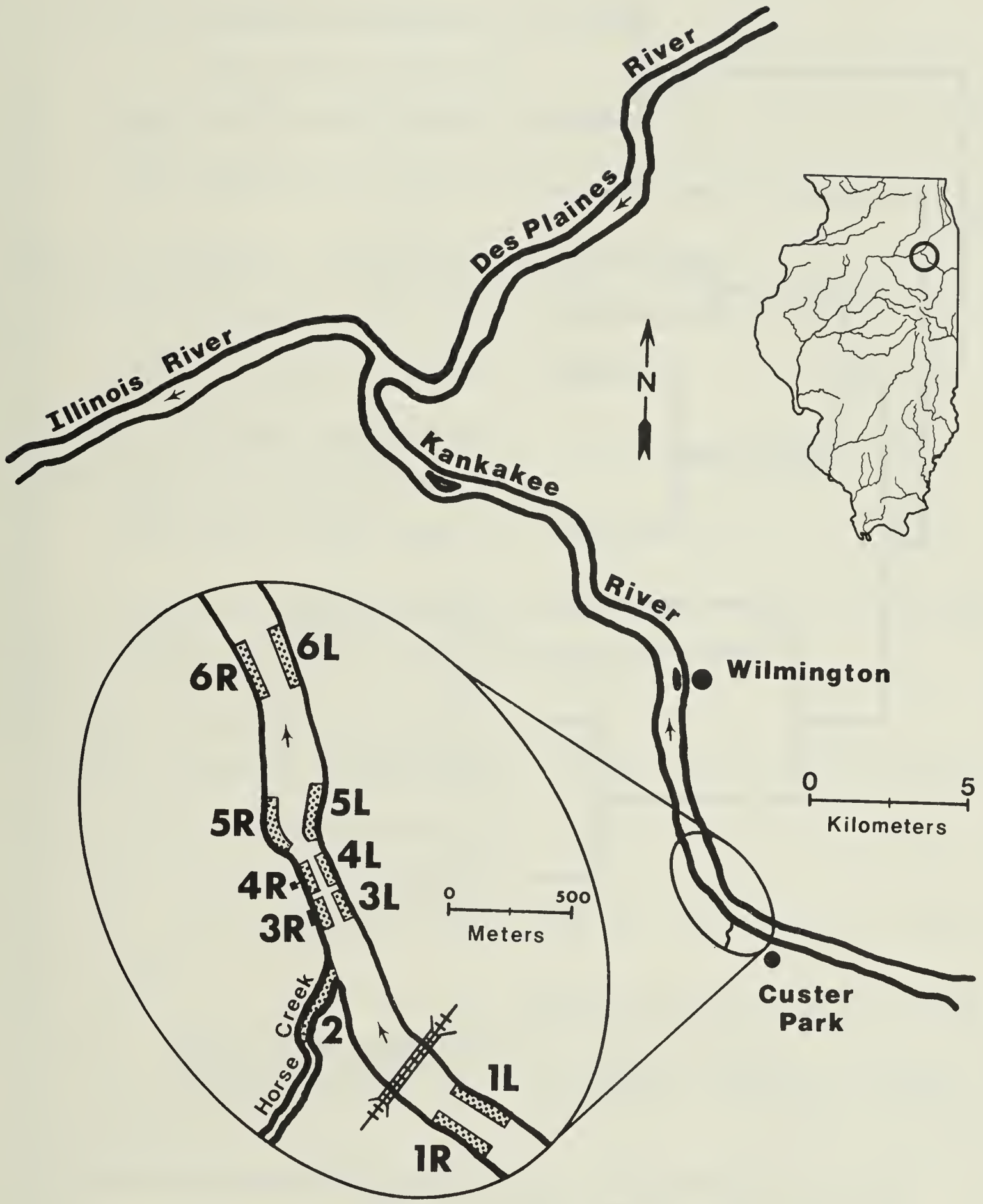

Fig. 1. Locations of sampling stations within the Braidwood Aquatic Monitoring Area of the Kankakee River.

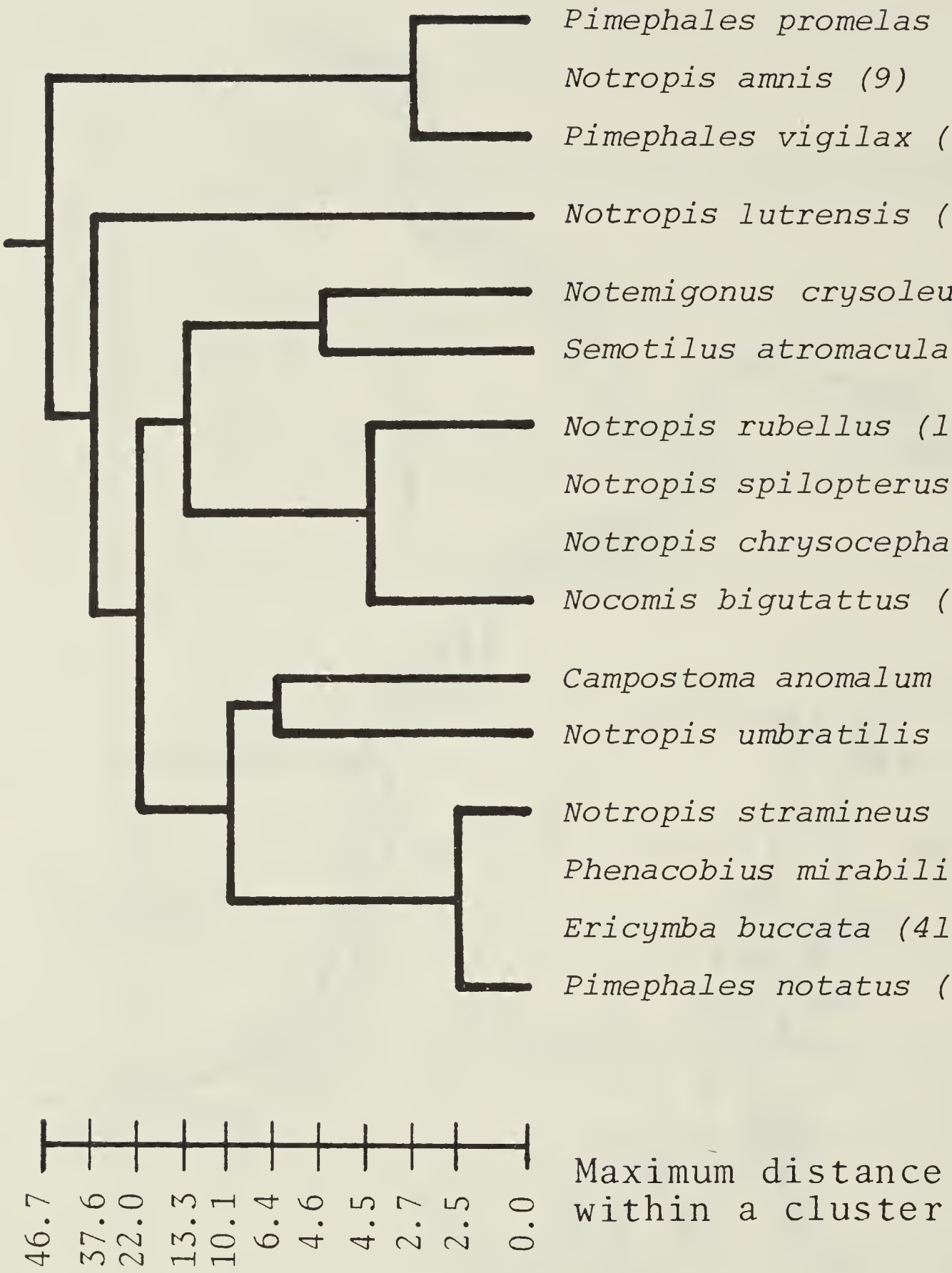

Fig. 2. Cluster analysis of Kankakee River cyprinid fishes by water quality parameters during August 1979. (N) = number of fishes included in the analysis.

Table 3. Number of fishes and percentages of the total catch by each species collected during a given month from four, 15-meter seine hauls at stations where *Notropis amnis* was collected.

	Nov. 1978 3R		Nov. 1978 5L		Aug. 1978 5R		Aug. 1979 5L		Aug. 1979 5R		Aug. 1981 5L		Aug. 1981 5R		Aug. 1982 5L	
	No.	% No.	No.	% No.	No.	% No.	No.	% No.	No.	% No.	No.	% No.	No.	% No.	No.	% No.
Dorosoma cepedianum	—	—	—	—	1	0.20	—	—	—	—	1	1.15	8	287	1	1.49
Esox americanus	—	—	1	0.81	—	—	—	—	—	—	1	1.15	2	0.72	—	—
Esox lucius	—	—	—	—	—	—	1	0.99	—	—	2	2.30	—	—	—	—
Campostoma anomalum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1.49
Cyprinus carpio	—	—	—	—	—	—	—	—	—	—	2	2.30	1	0.36	—	—
Ericymba buccata	—	—	—	—	3	0.60	—	—	3	0.60	—	—	—	—	—	—
Nocomis biguttatus	—	—	—	—	—	—	—	—	4	0.80	—	—	—	—	—	—
Notemigonus crysoleucas	—	—	2	1.63	—	—	6	5.94	—	—	1	1.15	—	—	4	5.97
Notropis amnis	1	0.04	2	1.63	1	0.20	4	3.96	5	1.00	2	2.30	1	0.36	2	2.99
N. buchanani	1	0.04	—	—	—	—	—	—	—	—	1	1.15	—	—	—	—
N. chrysocephalus	1	0.04	—	—	5	1.01	10	9.90	19	3.80	—	—	—	—	1	1.49
N. lutrensis	—	—	—	—	—	—	—	—	—	—	—	—	1	0.36	—	—
N. rubellus	43	1.85	—	—	1	0.20	12	11.88	24	4.80	—	—	—	—	—	—
N. spilopterus	994	42.72	5	407	13	2.62	16	15.84	207	41.40	7	8.05	14	5.02	1	1.49
N. stramineus	120	5.16	—	—	184	37.02	1	0.99	99	19.80	—	—	40	14.34	—	—
N. umbratilis	10	0.43	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. volucellus	60	2.58	—	—	—	—	—	—	—	—	1	1.15	4	1.43	—	—
Notropis spp.	12	0.52	—	—	2	0.40	—	—	—	—	—	—	—	—	—	—
Phenacobius mirabilis	—	—	—	—	6	1.21	—	—	17	3.40	1	1.15	13	4.66	—	—
Pimephales notatus	515	22.13	1	0.81	180	36.22	18	17.82	25	5.00	15	17.24	43	15.41	12	17.91
P. promelas	—	—	1	0.81	—	—	—	—	1	0.20	—	—	—	—	—	—
P. vigilax	567	24.37	67	54.47	44	8.85	3	2.97	36	7.20	6	6.90	96	34.41	—	—
Moxostoma anisurum	—	—	—	—	—	—	—	—	1	0.20	—	—	1	0.36	—	—
M. erythrurum	—	—	1	0.81	—	—	—	—	1	0.20	—	—	—	—	—	—
Moxostoma spp.	—	—	—	—	29	5.84	—	—	6	1.20	1	1.15	6	2.15	2	2.99
Fundulus notatus	—	—	—	—	1	0.20	1	0.99	—	—	—	—	1	0.36	1	1.49
Labidesthes sicculus	1	0.04	—	—	—	—	1	0.99	—	—	—	—	—	—	—	—
Ambloplites rupestris	—	—	1	0.81	—	—	—	—	—	—	—	—	1	0.36	—	—
Lepomis cyanellus	—	—	7	5.69	—	—	3	2.97	—	—	1	1.15	—	—	2	2.99
L. gibbosus	—	—	—	—	—	—	—	—	—	—	1	1.15	—	—	—	—
L. humilis	1	0.08	28	22.76	—	—	5	4.95	1	0.20	13	14.92	16	5.73	6	8.96
L. megalotis	—	—	—	—	3	0.60	—	—	—	—	3	3.45	11	3.94	—	—
Lepomis spp.	—	—	—	—	—	—	2	1.98	—	—	3	3.45	1	0.36	11	16.42
Micropterus dolomieui	—	—	—	—	—	—	3	2.97	5	1.00	—	—	7	2.51	—	—
M. salmoides	—	—	1	0.81	7	1.41	2	1.98	—	—	4	4.50	2	0.72	9	13.43
Pomoxis annularis	—	—	2	1.63	—	—	9	8.91	3	0.60	11	12.64	3	1.08	—	—
P. nigromaculatus	—	—	3	2.44	—	—	—	—	—	—	10	11.49	7	2.51	4	5.97
Etheostoma nigrum	—	—	1	0.81	14	2.82	3	2.97	36	7.20	—	—	—	—	8	11.94
Percina maculata	—	—	—	—	3	0.60	1	0.99	6	1.20	—	—	—	—	2	2.99
P. phoxocephala	—	—	—	—	—	—	—	—	1	0.20	—	—	—	—	—	—
No. individuals	2326		123		497		101		500		87		279		67	
No. species	12		15		15		18		19		19		20		14	

Table 4. Weighted mean water quality values for Kankakee River minnows collected on 9 and 15 August 1979.

Species	N	Vel. (cm/sec)	Temp (°C)	D.O. (mg/l)	Depth (m)	Turbidity N.T.U.	pH	Conductivity μ mhos/cm
Pimephales promelas	1	3.0	20.40	7.30	0.50	37.0	8.20	600
Notropis amnis	9	1.8	21.70	7.32	0.50	33.7	8.27	610
P. vigilax	62	3.7	21.78	7.12	0.63	33.2	8.30	620
N. lutrensis	2	2.0	26.90	6.40	1.00	28.0	8.60	700
Notemigonus crysoleucas	8	1.6	26.76	7.18	0.50	34.0	8.68	681
Semotilus atromaculatus	18	4.1	26.17	7.34	0.53	18.1	8.62	725
Notropis rubellus	139	6.9	25.61	6.41	0.51	35.8	8.49	679
N. spilopterus	1010	3.8	24.38	7.25	0.57	31.6	8.45	671
N. chrysocephalus	99	3.3	25.40	7.12	0.51	44.2	8.37	665
Nocomis biguttatus	19	3.2	24.16	6.80	0.61	40.5	8.39	654
Campostoma anomalum	2	10.0	26.20	8.00	0.50	18.1	8.62	725
Notropis umbratilis	7	12.7	25.84	7.14	0.50	24.7	8.40	707
N. stramineus	491	5.2	23.69	7.86	0.54	20.4	8.39	682
Phenacobius mirabilis	77	7.1	24.29	7.81	0.53	16.3	8.41	701
Ericymba buccata	41	7.2	23.94	8.27	0.50	10.1	8.40	708
Pimephales notatus	777	6.9	24.22	8.19	0.51	11.4	8.41	710

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TEMPERATURE PROFILES GENERATED BY A SOURCE TERM IN MOTION: A NON-LINEAR HEAT EQUATION

P. A. McCoy
Mathematics Department, United States Naval Academy
Annapolis, Maryland 21402

B. Friedman
David Taylor Naval Ship Research and Development Center
Annapolis, Maryland 21402

ABSTRACT: Applications in welding engineering require precise analytic models for temperature transmission through planar work pieces inorder to treat weldments for exotic materials such as titanium. The current state of the art views linear quasi-stationary problems for planar heat flow in workpieces whose physical parameters such as thermal conductivity are functions of position only. This investigation is an analytic approach to the solution of a non-linear planar heat flow problem generated from a distributional heat source moving along a smooth curve at constant speed. The quasi-stationary condition is not required and the thermal properties of the workpiece are functions of position and temperature. Thus, the problem is non-linear and time dependent. The solution follows by applying D'Almbert's transformation to the heat equation to generate a formally hyperbolic PDE in the complex-plane that is solved in equivalent form as a non-linear Volterra's integral equation. This produces an analtyic solution in the form of a rapidly converging series.

INTRODUCTION

Modern Technology requires the fabrication of a large number of metals, both familiar and exotic, under a variety of conditions. Mathematical studies of the heat flow process in a workpiece upon welding it are a valuable complement to experimental investigations because they are a significant aid in ascertaining the the more important parameters in determining the final quality of a weld. Such studies allow the construction of a framework within which to place experimental results.

According to Rosenthal [12] and Myers [10], interest in the study of the heat flow in a material upon the application of a moving heat source was originally motivated by electric arc welding. Rosenthal himself was the first investigator to apply the heat flow equation with a moving heat source to the study of welding and to obtain analytical solutions for some specific cases. All of the examples considered by Rosenthal involve constant linear

motion. The thermal properties, that is, the thermal conductivity and specific heat of the material were assumed constant and independent of the spatial coordinates, time and temperature. In order to simplify the heat equation, he applied a quasi-stationary condition. This condition was derived from experimental datum in which the temperature distribution around the heat source rapidly becomes time-independent if the region that is being heated is small compared to the entire body.

More complex cases than those of Rosenthal have been investigated by Des Ruisseaux and Zerkle [2], and by Kawashimo, Yamada, and Fujiwara [4]. They obtained analytical solutions for bodies with moving band sources that have cylindrically symmetric thermal properties. The motivation for those studies arose from machining processes.

The increasing need for more realistic mathematical studies of the welding process than Rosenthal's and the advent of the electronic computer have led to the utilization of numerical techniques for the solution of the heat flow equation with a moving heat source. The two main routes taken numerically have been the finite difference and the finite element methods. The numerical techniques allow the utilization of variable thermal properties in the calculations and do not require the imposition of the quasi-stationary condition. Westby [14] was the first investigator to apply the finite difference approach using a computer program. His work was refined and extended by Paley and Hibbert [11]. The application of the finite element approach was originated by Masubucki [9].

Increasing utilization of the computer suggests extension of the work initiated by Rosenthal in obtaining analytical solutions to the moving heat source problem in the more general non-linear cases. This investigation is an analytical approach to the solution of the non-linear two-dimensional heat flow equation with the point heat source moving along an arbitrary curve on the plane at a constant speed. The quasi-stationary condition is not required and the thermal properties of the workpiece are non-constant. Furthermore, the specific heat is analytic function of the spatial coordinates, the temperature, and the time. The thermal conductivity can be an analytic function of the spatial coordinates although it is constant with respect to both temperature and time. More precisely, the specific heat is an implicit function of the time because the time depends explicitly on the temperature and the specific heat is temperature dependent. Also, the two-dimensional planar heat flow models a "very" thin plate or a thick plate where the temperature flow in the z-direction is instantaneous.

A rationale of this extension of the work is that analytical solutions to a particular physical problem often yield greater insight into the physical mechanisms involved than do numerical solutions.

In the work described presently, the global solution for the temperature distribution follows by introducing a hypercomplex coordinate system (in analogy with the classical transformation of the wave equation by d'Alembert) that maps the non-linear heat equation into an equivalent hyperbolic partial differential equation. This equation is integrated to produce a non-linear Volterra integral equation that is solved by successive approximations. For other applications of these methods the reader my consult [1,3,6] and the references therein.

DERIVATION OF THE HEAT FLOW EQUATION

Consider the heat flow equation for a two-dimensional plane in its most general form:

$$(1) \qquad (\partial E/\partial \mu)(\partial \mu/\partial t) - \vec{\nabla} \cdot [K(x,y,\mu(x,y,t))\vec{\nabla}\mu] = (\partial Q/\partial t)$$

The factor $(\partial E(x,y,\mu(x,y,t))/\partial \mu) = \rho c = \rho(x,y,\mu(x,y,t))c(x,y,\mu(x,y,t))$ is the heat capacity of the material where ρ is the mass density and c is the specific heat.

The specific heat of a material for a given temperature $\mu(x,y,t)$ is defined by δE, the amount of heat required to raise the temperature of a unit mass of that material from μ to $\mu + \delta\mu$ where $\delta\mu$ is negligible compared to μ. The factor $K = K(x,y,\mu(x,y,t))$ is the thermal conductivity of the material and the term $(\delta Q(x,y,t)/\delta t)$ represents heat generated by sources internal to the material. In this investigation the internal heat source is a function of position only requiring that $(\partial Q/\partial t) \equiv 0$. We proceed by expanding the second term of the left-hand side of equation (1),

$$(2) \qquad \rho(x,y,\mu(x,y,t))c(x,y,\mu)(\partial \mu/\partial t) - \vec{\nabla} \cdot [K(x,y,\mu(x,y,t))\vec{\nabla}\mu]$$

In equation (2)

$$(3a) \qquad \vec{\nabla}K = (K_x + K_\mu \mu_x)\,\hat{i} + (K_y + K_\mu \mu_y)\hat{j}$$

and

$$(3b) \qquad \vec{\nabla}\mu = \mu_x \hat{i} + \mu_y \hat{j},$$

where

$$(3c) \qquad K_x = (\partial K/\partial x),\ K_\mu = (\partial K/\partial \mu),\ \mu_x = (\partial \mu/\partial x),$$

$$K_y = (\partial K/\partial y),\ \text{and}\ \mu_y = (\partial \mu/\partial y)$$

Utilizing equations (3a-c) yields

(3c) $$\nabla\mu\cdot\nabla K = K_x\mu_x + K_\mu\mu_x^2 + K_y\mu_y + K_\mu\mu_y^2$$

The equation (2) is now put into the form

(4) $$\rho(x,y,\mu)c(x,y,\mu)\mu_t - K(x,y,\mu)[\mu_{xx} + \mu_{yy}] - [K_x\mu_x + K_y\mu_y] - K_\mu[\mu_x^2 + \mu_y^2] = 0$$

where $\mu_{xx} = (\partial^2\mu/\partial x^2)$, $\mu_{yy} = (\partial^2\mu/\partial y^2)$ so that utilizing equations (3a-c) in equation (4) gives

(5) $$\rho(x,y,\mu)c(x,y,\mu)(\partial\mu/\partial t) = K(x,y,\mu)\nabla^2\mu + \vec{\nabla K}\cdot\vec{\nabla\mu} + K_\mu|\vec{\nabla\mu}|^2.$$

Let the heat source move at constant speed along a smooth curve in the plane and let (ζ,η) be the coordinates of a point on the curve that locates the heat source relative to the (stationary) origin. The coordinates (ζ,η) are functions of the arc length parameters. The coordinates (σ,τ) are the coordinates for a point in the plane relative to the heat source. Let MC denote a moving coordinate system which has its origin at the heat source with coordinates (ζ,η) and LC represents a laboratory coordinate system whose origin is stationary. The coordinates (x,y) in the LC system denote the same point (σ,τ) in the MC system. The LC and MC systems are related by

(6) $$\sigma = x - \zeta(s), \tau = y - \eta(s).$$

The transformation of the temperature between these coordinate systems is

$$(\partial\mu/\partial t)_{LC} = (\partial\mu/\partial t)_{MC} + (\partial\mu/\partial\sigma)(\partial\sigma/\partial\zeta)(d\zeta/ds)(ds/dt) +$$

(7a) $$(d\mu/\partial\tau)(\partial\tau/\partial\eta)(d\eta/ds)(ds/dt),$$

or since,

$$(d\sigma/ds) = (\partial\sigma/\partial\zeta)(d\zeta/ds), (d\tau/ds) = (\partial\tau/\partial\eta)(d\eta/ds),$$

(7b) $$(\partial\mu/\partial t)_{LC} = (\partial\mu/\partial t)_{MC} + (\partial\mu/\partial\sigma)(d\sigma/ds)(ds/dt) + (\partial\mu/\partial\tau)(d\tau/ds)(ds/dt).$$

Now, the inverses:

$$(d\sigma/ds) = (ds/d\sigma)^{-1}, (d\tau/ds) = (ds/d\tau)^{-1}$$

exist provided that the curve is smooth

(8a) $$|d\sigma/ds| \leq M, |d\tau/ds| \leq M$$

for some positive constant M. From eq. (6), it is seen that the parameter s itself is a function of the moving coordinates (σ,τ). Then,

(8b) $$(d\sigma/ds) = (1/\dot{s}(\sigma)),\ (d\tau/ds) = (1/\dot{s}(\tau)),$$

where

$$\dot{s}(\sigma)=(ds(\sigma)/d\sigma),\dot{s}(\tau) = (ds(\tau)/d\tau).$$

Equation (7b) then becomes

(9) $$(\partial\mu/\partial t)_{LC} = (\partial\mu/\partial t)_{MC} + [(\partial\mu/\partial\sigma)(1/\dot{s}(\sigma)) + (\partial\mu/\partial\tau)(1/\dot{s}(\tau))](ds/dt)$$

We require that the speed of the heat source $|ds/dt|$ be a constant with a value of ν, so that equation (9) becomes

(10) $$(\partial\mu/\partial)_{LC} = (\partial\mu/\partial t)_{MC} + (\nu/\dot{s}(\sigma))(\partial\mu/\partial\sigma)+(\nu/\dot{s}(\tau))(\partial\mu/\partial\tau).$$

Substitution of equation (10) into equation (5) yields

$$\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)[(\partial\mu/\partial t)_{MC} + (\nu/\dot{s}(\sigma))(\partial\mu/\partial\sigma) + (\nu/\dot{s}(\tau))(\partial\mu/\partial\tau)]$$

(11) $$= K(\sigma,\tau,\mu)[(\partial^2\mu/\partial\sigma^2) + (\partial^2\mu/\partial\tau^2)] + K_\sigma\mu_\sigma + K_\tau\mu_\tau + K_\mu[\mu_\sigma^2 + \mu_\tau^2].$$

Upon rearrangement equation (11) becomes

$$\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)(\partial\mu/\partial t) = K(\sigma,\tau,\mu)[\mu_{\sigma\sigma} + \mu_{\tau\tau}] = [K_\sigma - (\nu/\dot{s}(\sigma))c(\sigma,\tau,\mu)]\mu_\sigma$$

(12) $$+ [K_\tau - (\nu/\dot{s}(\tau))c(\sigma,\tau,\mu)]\mu_\tau + K[\mu_\sigma^2 + \mu_\tau^2],$$

where the subscript MC has been dropped. Now, assuming that $K_\mu \equiv 0$, so that the thermal conductivity is independent of temperature reduces equation (12) to

$$\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)\partial\mu/\partial t = K(\sigma,\tau)[\mu_{\sigma\sigma} + \mu_{\tau\tau}]$$

(13) $$+ [K_\sigma - (\nu/\dot{s}(\sigma))c(\sigma,\tau,\mu)]\mu_\sigma + [K_\tau - (\nu/\dot{s}(\tau))c(\sigma,\tau,\mu)]\mu_\tau.$$

Normalization of equation (13) gives

$$d(\sigma,\tau,\mu)(\partial\mu/\partial t) =$$

(14) $$(\partial^2\mu/\partial\sigma^2) + (\partial^2\mu/\partial\tau^2) + a(\sigma,\tau,\mu)(\partial\mu/\partial\sigma) + b(\sigma,\tau,\mu)(\partial\mu/\partial\tau)$$

where

(15a) $$d(\sigma,\tau,\mu) \equiv (\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)/K(\sigma,\tau)),$$

(15b) $$a(\sigma,\tau,\mu)\equiv(1/K(\sigma,\tau))[K_\sigma - (\nu/\dot{s}(\sigma))\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)],$$

(15c) $$b(\sigma,\tau,\mu)\equiv(1/K(\sigma,\tau))[K_\tau -(\nu/\dot{s}(\tau))\rho(\sigma,\tau,\mu)c(\sigma,\tau,\mu)].$$

SOLUTION OF THE HEAT FLOW EQUATION

The real variables (σ,τ) are now continued as independent complex-variables from which the hyper-complex coordinates

(16) $$\sigma = (z + z^*)/2,\ \tau = (z - z^*)/(2i)$$

are defined. In the event that σ and τ are real, then z^* is the complex conjugate of z. In other words, $z^* = z$ if, and only if, (σ,τ) are real.

Requiring the specific heat and thermal conductivity to be analytic on $C^3 = CxCxC$ (C being the complex-plane), equation (14) becomes formally a hyperbolic equation

(17a) $$U_{zz^*} - A(z,z^*,U)U_z - B(z,z^*,U)U_{z^*} = D(z,z^*,U)U_t,$$

in the hyper-complex system with coefficients

(17b) $$A = -1/4(a+ib), B = -1/4(a-ib), D = 1/4d.$$

After rearranging, equation (17a) becomes

(18) $$U_{zz}^* = D(z,z^*,U)U_t + A(z,z^*,U)U_z + B(z,z^*,U)U_{z^*}.$$

Equation (18) is now integrated to produce the (non-linear) Volterra integral equation

(19a) $$U(z,z^*,t) = \int_0^z \int_0^{z^*} H(w,w^*,U)dw^*dw,$$

where

(19b) $$H(w,w^*,U) = D(w,w^*,U)U_t + A(w,w^*,U)U_w + B(w,w^*,U)U_w^*.$$

Equation (19a) is to be solved by successive iterations. Thus for $n \geq 1$,

$$A^{(n-1)} = A^{(n-1)}(z,z^*,U^{(n-1)}) = A^{(n-1)}(z,z^*),$$

$$B^{(n-1)} = B^{(n-1)}(z,z^*,U^{(n-1)}) = B^{(n-1)}(z,z^*)$$

$$D^{(n-1)} = D^{(n-1)}(z,z^*,U^{(n-1)}) = D^{(n-1)}(z,z^*) \tag{20}$$

In general, $A^{(0)}$, $B^{(0)}$, $C^{(0)}$ are arbitrary constants. Equation (18) is then solved for $U^{(0)}$ by the methods of Bergman and Gilbert [9], [10]. However, a natural choice for our problem is $U^{(0)} = U^{(0)}(z,z^*,t)$, the ambient temperature profile in the plate.

The iterated form of equation (19a) is, for $n \geq 1$,

$$U^{(n)}(z,z^*,t) = \int_0^z \int_0^{z^*} H^{(n-1)}(w,w^*,U^{(n-1)})dw^*dw, \tag{21a}$$

where

$$H^{(n-1)}(w,w^*,U^{(n-1)}) = D^{(n-1)}(w,w^*,U^{(n-1)})U^{(n-1)}$$

$$+ A^{(n-1)}(w,w^*,U^{(n-1)})U_w^{(n-1)} + B^{(n-1)}(w,w^*,U^{(n-1)})U_{w^*}^{(n-1)} \tag{21b}$$

The validity of the iterative approach is dependent on the absolute and uniform convergence of the telescoping series

$$(U^{(0)} - U^{(1)}) + \sum_{k=1}^{n} W^{(k+1)} = (U^{(0)} - U^{(1)}) + (U^{(1)} - U^{(2)}) + \ldots + (U^{(n-1)} - U^{(n)})$$

$$= U^{(0)} + U^{(n+1)} \tag{22a}$$

where

$$W^{(k+1)} = U^{(k-1)} - U^{(k)}, \quad k = 1,2,\ldots,n \tag{22b}$$

The demonstration of the convergence commences with the series

$$\sum_{n=0}^{\infty} W^{(n+1)} = \sum_{n=0}^{\infty} (U^{(n-1)} - U^{(n)}) \tag{23a}$$

where

$$W^{(n+1)} = \int_0^z \int_0^{z^*} [H^{(n+1)}(w,w^*,U^{(n+1)}) - H^{(n)}(w,w^*,U^{(n)})]dwdw^*, \tag{23b}$$

and

$$\int_0^z \int_0^{z^*} [H^{(n+1)}(w,w^*,U^{(n+1)}) - H^{(n)}(w,w^*,U^{(n)})]\,dw\,dw^*$$

$$= \int_0^z \int_0^{z^*} (F^{(n+1)} + G^{(n+1)} + L^{(n+1)})\,dw^*\,dw,$$

$$F^{(n+1)} = [D^{(n+1)}(w,w^*,U^{(n+1)})U_t^{(n+1)} - D^{(n)}(w,w^*,U^{(n)})U_t^{(n)}]$$

$$G^{(n+1)} = [A^{(n+1)}(w,w^*,U^{(n+1)})U_w^{(n+1)} - A^{(n)}(w,w^*,U^{(n)})U_w^{(n)}],$$

$$L^{(n+1)} = [B^{(n+1)}(w,w^*,U^{(n+1)})U_{w^*}^{(n+1)} - B^{(n)}(w,w^*,U^{(n)})U_{w^*}^{(n+1)}]$$

Let

$$M = \max\{|D^{(n+1)}|, |D^{(n)}|, |A^{(n+1)}|, |A^{(n)}|, |B^{(n+1)}|, |B^{(n)}|\},\ n \geq 1$$

As we seek the analytic solution, the constant is finite for the arguments of the functions on compact simply connected sets in C^3. From equation (23b),

$$|W^{(n+1)}| \leq \int_0^z \int_0^{z^*} [|F^{(n+1)}| + |G^{(n+1)}| + |L^{(n+1)}|]\ dw\,dw^*,\ n \geq 0$$

Now consider the first term in the integrand of equation (24), (reducing the index n by 1)

$$|F^{(n+1)}| \leq \int_0^z \int_0^{z^*} |F^{(n)}|\,dw\,dw^*,\ n \geq 0.$$

Let

$$K_1 = \max|U_t^{(1)} - U_t^{(0)}|$$

$$|F^{(2)}| \leq \int_0^z \int_0^{z^*} |F^{(1)}|\,dw\,dw^* \leq MK_1 \int_0^z \int_0^{z^*} dw\,dw^* = MK_1\ zz^*.$$

Similarly,

$$|F^{(3)}| \leq \int_0^z \int_0^{z^*} |F^{(2)}|\,dw\,dw^* \leq MK_1 \int_0^z \int_0^{z^*} ww^*\,dw^*\,dw = MK_1 \frac{z^2}{2!} \frac{(z^*)^2}{2!}$$

so that by induction

$$|F^{(n)}| \leq MK_1 \frac{z^n(z^*)^n}{n!n!}, \quad n \geq 1,$$

Likewise:

$$|G^{(n)}| \leq MK_2 \frac{z^n(z^*)^n}{n!n!}, \quad n \geq 1,$$

$$|L^{(n)}| \leq MK_3 \frac{z^n(z^*)^n}{n!n!}, \quad n \geq 1,$$

Where K_2 and K_3 are defined similarly to K_1.

Substituting the results of equations (25a-c) into equation (24) yields

$$|W^{(n+1)}| \leq M(K_1 + K_2 + K_3) \int_0^z \int_0^{z^*} \frac{w^{(n+1)}(w^*)^{n+1}}{(n+1)!(n+1)!} dw^*dw$$

$$= M(K_1 + K_2 + K_3) \frac{z^{n+2}(z^*)^{n+2}}{(n+2)!(n+2)!}, \quad n \geq -1.$$

Thus, the series

$$\left| \sum_{n=0}^{\infty} W^{(n+1)} \right| \leq \sum_{n=0}^{\infty} |W^{(n+1)}| \leq M(K_1 + K_2 + K_3) \sum_{n=0}^{\infty} \frac{|z|^{n+2}|z^*|^{n+2}}{(n+2)!(n+2)}$$

is absolutely and uniformly convergent for all compact subsets of C^2 $X[0,t_0]$.

Consequently, the telescoping series equation (22a) is absolutely and uniformly convergent on simply connected compact sets of C^2 $X[0,t_0]$. This means that the function

$$U(z,z^*,t) = U^{(0)}(z,z^*,t) - \lim_{n\to\infty} U^{(n+1)}(z,z^*,t) \tag{26}$$

represents the analytic solution on compact sets of C^2 $X[0,t_0]$. In other words, a global solution. The error in truncating the series equation (22a) for an approximate solution of $(n+1)$ terms is less then $O(R^n(R^*)^n/((n+1)!)^2)$ for $|z| < R$, $|z^*| < R^*$. Moreover, the complex valued solution in real coordinates (σ,τ) is recovered by inverting the transformation equation (16) as

$$z = \sigma + i\tau, \quad z^* = \sigma - i\tau$$

The real solution is the real part of the complex-valued solution.

Let us now consider an elementary example of this technique. The heat source is travelling at constant speed ν along the straight line: $\zeta = \alpha s$, $\eta = \beta s$ where α and β are constants. The density ρ, specific heat c and the thermal conductivity K are constants. In real coordinates, the heat flow equation is

$$\rho c \mu_t = K[\mu_{\sigma\sigma} + \mu_{\tau\tau}] + c\nu(\alpha\mu_\sigma + \beta\mu_\tau). \tag{27}$$

The coefficients $A^{(n)}$, $B^{(n)}$, $D^{(n)}$ are constants for all values of n and are given the constant values ℓ, m , j, respectively, where

(28) $\ell = (1/4\nu)(\rho c/K)(\alpha + \beta i)$, $m = (-1/4\nu)(\rho c/K)(\alpha - \beta i)$, $j = (1/4)(c/K)$.

In equation (26), the relationship between $U^{(n)}$ and $U^{(n+1)}$ is given by (neglecting the time dependence)

$$U^{(n+1)}(z,z^*) = (\ell L_1 + mL_2)U^{(n)}(z,z^*), \tag{29}$$

where L_1 and L_2 are operators such that

$$L_1 U^{(n)}(z,z^*) = \int_0^{z^*} U^{(n)}(z,w^*)dw^*,$$

$$L_2 U^{(n)}(z,z^*) = \int_0^{z} U^{(n)}(w,z^*)dw \tag{30}$$

Utilization of equation (29) gives

$$U^{(n+1)} = (\ell L_1 + mL_2)^{n+1} U^{(0)}. \tag{31}$$

Take $\ell = m$, and $r = \ell(L_1 + L_2)$. Then

$$U(z,z^*) = U^{(0)}(z,z^*) - \lim_{n\to\infty} r^{n+1}U^{(0)}(w,w^*), \text{ or} \tag{32a}$$

$$U(z,z^*) = U^{(0)}(z,z^*) - \lim_{n\to\infty} (-1 + (1-r)(1+r+r^2+ \ldots +r^n))U^{(0)}(w,w^*). \tag{32b}$$

Consider the case where the temperature distribution in the complex-plane is the Gaussian distribution

$$\mu^{(0)}(\sigma,\tau) = T_o e^{-(\sigma^2+\tau^2)/\varepsilon},$$

where ε is a parameter that defines the "diffuseness" of the heat source.

The zero-order approximation is $\mu(\sigma,\tau)_{n=0} = T_o e^{-(\sigma^2 + \tau^2)/\varepsilon} - T_o \ell \sqrt{\varepsilon\pi}$

$$[e^{-\tau^2/e}\ \Phi_0(\sigma\varepsilon/\ \frac{2}{\varepsilon}\ ) + e^{-\sigma^2/\varepsilon}\ \Phi_0(\tau/\ \frac{2}{\varepsilon}\ )]$$

where $\Phi_0(\xi)$ is the probability integral $\frac{1}{\sqrt{2\pi}} \int_0^{\xi} e^{-t^2/2}\, dt.$

Runs have been made for aluminum and titanium under various conditions. The "bow wave" phenomena, a heat flow analogy to the "sonic boom", which was noted in previous experiments and calculations [12] was observed.

This phenomenon demonstrates that the peak temperature on the plane occurs ahead of the heat source. For aluminum:

$$\rho=2.70g/cc(20°C),c=0.215cal/g^{-1}{}^{0}K^{-1}(25°C),K=2.37watts/cm\ °C(25°C).$$

For titanium:

$$\rho=4.5g/cc,c=0.125cal/g\ °C(25°C),K=0.2watts/cm\ °CM(25°C).[12]$$

CONCLUDING REMARKS

The work presented herein constitutes a generalization of the work of Rosenthal [12] for the heat flow in a material where the heat source is moving with constant speed in a straight line on a plane. In this investigation, the results do not require the assumption of a quasi-stationary condition. This allows the investigation of transient processes while a workpiece is being welded. The specific heat and thermal conductivity are not required to be constant although the thermal conductivity is required to be independent of the temperature. The heat source is allowed to move along any smooth curve in the plane.

As noted earlier, the results presented herein apply to "very" thin plates. These results can also be meaningfully interpreted for heat flows with cylindrical symmetry in the mathematical sense. Consider a solid extending to $\pm\infty$ in the z direction. In this event, all of the functions $\Phi(x,y,t) = \Phi(x,y,0,t) = \Phi(x,y,z,t)$ assume constant values along lines parallel to the 0-axis.

Further, it must be noted that our results are valid only when the velocity of the heat source is less than the rate of heat conduction. This means that these results are a function of the ratio of the velocity of the heat source to the thermal conductivity. Analytic solutions may not be possible for the case when the velocity of the heat source is greater than the rate of heat conduction because of the apparent singularity that builds up The velocity of the propagation of the heat as a "bow wave." conduction is given by the square root of the ratio of the thermal diffisivity and relaxation time [7]. For a metal, such as aluminum, this relaxation time is of the order of 10^{-11}sec[13]. For aluminum, at 0°C, the thermal diffusivity is approximately $10^{-3}ft^2/sec$ [14]. The velocity of propagation is then approximately 10^4ft/sec a velocity which is far greater han the maximum anticipated speed of the heat source of 3ft/sec in electric arc welding. It is such welding which is the movitivation for this study.

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A NEW LOOK AT THE EIGENVALUE PROBLEM FOR REAL SYMMETRIC MATRICES

by

John Gregory and Don Redmond

Section 1. Introduction. In this paper, we will take a new look at the eigenvalue problem for real symmetric matrices. Although the basic results are well-known, we will present ideas and methods that are not usually associated with this subject nor with each other. Our goal is to present these ideas using elementary techniques.

We begin with some preliminary notation and results. We assume that the reader is familiar with the definition of $H = IR^n$ as a real vector space. We have the usual inner product on H: if $x = (a_1, \ldots, a_n)$ and $y = (b_1, \ldots, b_n)$, then

$$(x,y) = \sum_{j=1}^{n} a_j b_j = y^T \cdot x,$$

where y^T is the transpose of the vector y. This induces the norm on H

$$\|x\| = \sqrt{(x,x)}.$$

A is a real symmetric matrix if $a_{ij} = a_{ji}$. In this case the associated quadratic form is $Q(x) = (Ax,x) = x^TAx$, for $x \in H$. The associated bilinear form is $Q(x,y) = (Ax,y) = y^TAx$. It is immediate $Q(x,y) = Q(y,x)$ for x,y in H and that there is a one to one, onto mapping (isomorphism) between the collection of symmetric matrices and the collection of quadratic forms.

The principal result we will need in what follows in the so-called principal axiom theorem. Recall that an orthogonal matrix P is one satisfying $P^{-1} = p^T$. Theorem 1 may be found in [2, p. 266].

THEOREM 1. If A is a real symmetric matrix, then there exists an orthogonal matrix P such that $p^TAP = D$, where D is a real diagonal matrix.

Note that if $D = \text{diag}\{\lambda_1, \ldots, \lambda_n\}$, then $\lambda_2, \ldots, \lambda_n$ are the eigenvalues of A. In terms of the associated quadratic form this gives, if $A = (a_{ij})$

$$\sum_{i,j=1}^{n} a_{ij}x_ix_j = Q(x) = (Ax,x) = x^TAx = x^TPDP^Tx = (P^Ts)^TD(P^Tx) = y^Tdy = \sum_{i=1}^{n} \lambda_i y_i^2.$$

Thus, by a change of basis, which in this case amounts to a rotation of the axes, we can obtain diagonal quadratic forms, providing we know the eigenvalues of the matrix A.

Section 2. Rayleigh-Ritz Method. One of the standard techniques for obtaining the eigenvalues of a real symmetric matrix A, or the corresponding quadratic form Q(x), is the Rayleight-Ritz procedure. The Rayleigh quotient of A is defined to be

$$R(x) = \frac{(Ax,x)}{(x,x)} = \frac{Q(x)}{\|x\|^2} \quad \text{for } x \neq 0.$$

The eigenvalues of A (or Q(x)), then turn out to be maximums (or as we shall see minimum) of R(x) taken over an appropriate subspace of $\mathbb{R}^n - \{0\}$. Indeed we have the following result. (See [1, pg. 78]).

THEOREM 2. If A is a symmetric matrix, there exists an orthonormal set of eigenvectors $\{x_1, x_2, \ldots, x_n\}$ of A such that the corresponding eigenvalue $\{\lambda_1, \lambda_2, \ldots, \lambda_n\}$ satisfy $\lambda_k = R(x_k)$ and $\lambda_1 \leq \lambda_2 \leq \ldots \leq \lambda_n$. The vector x_1 minimizes R(x) on $\mathbb{R}^n - \{0\}$ and the vector x_n maximizes R(x) on $\mathbb{R}^n - \{0\}$. For each k such that $1 < k < n$, the vector x_k minimizes R(x) on the set

$$B_k = \{x \in \mathbb{R}^n : (x,x_i) = 0,\ 1 \leq i \leq k-1\} - \{0\}$$

$$= \text{span}\ \{x_k, \ldots, x_n\} - \{0\}.$$

Similarly x_k maximizes R(x) on the set

$$C_k = \{x \in \mathbb{R}^n : (x,x_i) = 0,\ k+1 \leq i \leq n\} - \{0\}$$

$$= \text{span}\ \{x_1, \ldots, x_{k-1}\} - \{0\}.$$

Finally, if D^k denotes a subspace of dimension k without x = 0 and $\overline{D}^k$ denotes the collection of all such spaces, then λ_k satisfies the following (min-max or max-min) principal

$$(1) \qquad \lambda_k = \min_{\overline{D}^k}\{\max_{x\in D^k} R(x)\} = \max_{D^{n-k+1}}\{\min_{x\in D^{n-k+1}} R(x)\}.$$

This last equality, (1), seems particularly formidable. Since B_k is one of the sets D^{n-k+1}, we have, by an earlier part of the theorem

$$\min_{x\in D^{n-k+1}} R(x) \leq \min_{x\in D_k} R(x) = \lambda_k,$$

but since λ_k is "obtained" we may maximize both sides to obtain the max-min equality in (1). The first equality in (1) follows similarly or one may follow the suggestion of Hestenes [1] and apply this argument to the eigenvalues of -A, which are $-\lambda_n \leq \cdots \leq -\lambda_1$.

We can give an easy heurestic proof of Theorem 2 as follows. By Theorem 1 we may diagonalize A, that is, we may find an orthogonal matrix P such that

$$\Lambda = P^T A P = \mathrm{diag}(\lambda_1, \ldots, \lambda_n),$$

where we have assumed $\lambda_1 \leq \ldots \leq \lambda_n$. If $e_1, \ldots, e_n$ are the standard unit vectors, then they are the eigenvectors of A and we hence $Q(e_k) = \lambda_k$. If $x = (c_1, \ldots, c_n)^T$ is such that

$$\|x\|^2 = \sum_{i=1}^{n} c_i^2 = 1,$$

then $R(x) = \sum_{i=1}^{n} \lambda_i c_i^2$ has the smallest value, when $c_1 = 1$ and $c_k = 0$ for $k = 2, \ldots, n$. To see this, note that R(x) is a convex combination of the $\{\lambda_i\}$ and

$$S = \{(\lambda_1 d_1, \ldots, \lambda_n d_n) \ : \ d_i \geq 0, \ \sum_{i=1}^{n} d_i = 1\}$$

is the face or the intersection of the n - 1 dimensional hyperplane determined by $\{\lambda_i\}$ and the positive "octant" in n space. In the above $c_i^2 = d_i$, $1 \leq i \leq n$. In fact R(x) is the sum of the coordinates on the S above. In linear programming terminology we wish to minimize $f(t) = t_1 + \ldots + t_n$, where $t = (t_1, \ldots, t_n)^T$ is in S. Since S is a convex set and f is linear, the minimum value of f on S exists and occurs at "corner" points of S. An immediate calculation gives the desired result since the extreme points are on the coordinate axes.

Figure 1 illustrates these ideas with m = 3 and $\lambda_i > 0$, since the geometric ideas hold under the translation $s_i = |\lambda_n| + t_i$. In this case, $\min\{R(x) \ : \ x \in S\} = \lambda_1$ as stated. Similarly (in this picture) S_1 in the set $\{(0_1 a_1, a_2)\}$, where (a_1, a_2) lie on the line segment connecting λ_2 and λ_3. More generally the set S above becomes an n - 2 dimensional "face"

$$S_1 = \{x \in S : d_1 = 0\}$$

for the "λ_2-problem." Once again $\lambda_2 = \min\{R(x) \ : \ x \in S_1\}$. The similar argument holds for λ_k, $2 \leq k \leq n$, by constructing a collection $S, S_1, S_2, \ldots, S_{n-1}$ where each S_{i+1} is the "positive" edge of S_i. In Figure 1, S_1 is the positive edge of the face S.

Note that if a is a nonzero real number, then

$$R(ax) = Q(ax)/\|ax\|^2 = \frac{a^2 Q(x)}{a^2 \|x\|^2} = R(x).$$

Thus R is homogeneous of degree 0. Pick D^k in $\overline{D}^k$. We may search for optimal values of the component function on S (by the homogeneity of R). The value max R(x), x in D^k, is taken on at the intersection point $\underline{P}$ of an edge S and D^k, since, as above, extremal values of a linear function cannot occur in the interior of S_1. By the above argument, if $\underline{P}$ is not a corner or extreme point, it is not optimal. The minimum of such values occurs when D^k = space $\{e_1, \ldots, e_n\}$.

Finally, the problems of finding optimal values of A and Λ are

are equivalent, since P is one to one and preserves lengths. For example, $e_1 = (1, 0, \ldots, 0)^T$ satisfies

$$\lambda_1 = (\Lambda e_1, e_1) = \max_{x\neq 0} \frac{(\Lambda x, x)}{(x,x)} = \max_{x\neq 0} \frac{(P^T A x, Px)}{(x,x)} = \max_{x\neq 0} \frac{(APx, Px)}{(x,x)}$$

$$= \max_{y\neq 0} \frac{Ay, y}{(P^T y, 0^T y)} = \max_{y\neq 0} \frac{(Ay, y)}{(y,y)}$$

since $(P^T y, P^T y) = (y, PP^T y) = (y,y)$. Thus Theorem 2 is a "rotation", or more correctly an isometry (distance preserving mapping) of an apparent geometrical picture.

The remainder of this section is partly out of order developmentally with Theorem 2. That is, we might have placed this material before Theorem 2. We have included this material to show interesting calculations and concepts for quadratic forms. The following theorem is stated in Hestenes [1, p. 73]. Of interest are both the results and the computations involved in obtaining these results.

THEOREM 3. A vector x is an eigenvector of A if and only if it is a critical point of R(x). The eigenvalues of A are the corresponding critical values.

Let x_0 be a critical point of R(x) with $\lambda = R(x_0) = Q(x_0)/\|x_0\|^2 = (Ax_0, x_0)/(x_0, x_0)$ the critical value. Then for any vector and $\varepsilon > 0$ and small we have

$$\frac{1}{\varepsilon}\left[\frac{Q(x_0 + \varepsilon y)}{\|x_0 + \varepsilon y\|^2} - \frac{Q(x_0)}{\|x_0\|^2}\right] =$$

$$= \frac{1}{\varepsilon} \frac{\|x_0\|^2[Q(x_0) + 2\varepsilon Q(x_0,y) + Q(y)] - Q(x_0)\ [\|x_0\|^2 + 2\varepsilon(x_0,y) + \|y\|^2]}{\|x_0 + \varepsilon y\|^2 \|x_0\|^2}$$

$$= 2 \frac{\|x_0\|^2 Q(x_0,y) - Q(x_0)\ (x_0,y)}{\|x_0 + \varepsilon y\|^2 \|x_0\|^2} + \varepsilon \frac{\|x_0\| Q(y) - Q(x_0)\|y\|^2}{\|x_0 + \varepsilon y\|^2 \|x_0\|^2}$$

Taking the limit as $\varepsilon \to 0$ and noting that $Q(x_0) = \lambda\|x_0\|^2$ we have

$$\lim_{\varepsilon\to 0} \frac{1}{\varepsilon} [R(x_0 + \varepsilon y) - R(x_0)] = \frac{2}{\|x_0\|^2} [Q(x_0,y) - \lambda(x_0,y)].$$

This limit is zero for all y if and only if $Q(x_0,y) - (x_0,y) = 0$ or $(Ax_0 - \lambda x_0, y) = 0$ for all y, i.e., $Ax_0 = \lambda x_0$. Thus our result follows.

Some comments are in order. We have $R(x + \varepsilon y) = R(x) + R'(x,\varepsilon y) + \frac{1}{2}R''(x,\varepsilon y) + \ldots$, where $R'(x,h) = \nabla R(x)h$ and $R''(x,h) = h^T Ah$. R' is linear in its second argument and equal to $\nabla R \cdot (\varepsilon y)$ so that subtracting

$R(x)$ from both sides, dividing by ε and letting $\varepsilon \to 0$ we have $R'(X_0,y) = \frac{2}{\|x_0\|^2}[Q(x_0,y) - \lambda(x_0,y)]$ or the value of the gradient is $\nabla R(x_0) = \frac{2}{\|x_0\|^2}[Ax_0 - R(x_0)x_0]$. Locally at $x = x_0$, $R(x + \varepsilon y) - R(x)$ is linear in y if x_0 is not an eigenvector of A. If x_0 is an eigenvector of A, then this expression is locally quadratic in y.

It is illustrative to use elementary calculus to obtain the (first and) second directional derivatives of the Rayleigh quotient $R(x)$ and the Taylor series expansion as in (3). This will enable us to independently derive a "stronger" result than in Theorems 2 and 3. The critical point x_k is an <u>(ℓ,m) saddle point</u> of $R(x)$ if there exists subspaces S_1 and S_2 of $\mathbb{R}^n$ of dimension ℓ and m respectively such that $y_1 \neq 0$ in S_1 and $y_2 \neq 0$ in S_2 imply that there exists $\delta > 0$ such that $|\varepsilon| < \delta$ implies $R(x_k + \varepsilon y_1) < R(x_k) < R(x_k + \varepsilon y_2)$. The above means that locally we may decrease the critical value $\lambda_k = R(x_k)$ by "moving" from $x = x_k$ in an ℓ dimensional direction and increase $R(x_k)$ by "moving" from $x = x_k$ in an m dimensional direction In Theorem 4 we show that we may choose $S_1 = \text{span}\{x_1, \ldots, x_\ell\}$ and $S_2 = \text{span}\{x_{n-m+1}, \ldots, x_n\}$.

THEOREM 4. Let $\lambda_1 \leq \lambda_2 \leq \ldots < \lambda_n$ be the eigenvalues of A with corresponding eigenvectors $x_1, x_2, \ldots, x_n$ respectively. If for some k $(1 < k < n)$ we have $\lambda_1 < \lambda_k < \lambda_n$, then the critical point x_k is a saddle point of the Rayleigh quotient (neither a local maximum or minimum). More precisely, if $\lambda_\ell < \lambda_k < \lambda_{n-m+1}$ then x_k is an (ℓ,m) saddle point. Finally λ_1 and λ_n are respectively the absolute minimum and maximum of $R(x)$ on $\mathbb{R}^n - \{0\}$.

Let $h(\varepsilon) = Q(x + \varepsilon y)$, $g(\varepsilon) = \|x + \varepsilon y\|^2$, and $R(x + \varepsilon y) = f(\varepsilon) = h(\varepsilon)/g(\varepsilon)$. Now $f(\varepsilon) = f(0) + \varepsilon f'(0) + \frac{1}{2}\varepsilon^2 f''(0) + \ldots$ where $f'(\varepsilon) = [g(\varepsilon)h'(\varepsilon) - h(\varepsilon)g'(\varepsilon)]/g^2(\varepsilon)$ and

$$f''(\varepsilon) = \frac{g^2(\varepsilon)[g'(\varepsilon)h'(\varepsilon) + g(\varepsilon)h''(\varepsilon) - h'(\varepsilon)g'(\varepsilon) - h(\varepsilon)g''(\varepsilon)] - \{\ \}}{S^4(\varepsilon)}.$$

We have not bothered to determine $\{\ \}$ since it is zero when $\varepsilon = 0$ (a critical point). Thus $f'(0) = [g(0)h'(0) - h(0)g'(0)]/g^2(0)$ and $f''(0) = [g(0)h''(0) - h(0)g''(0)]/g^2(0)$.

The first and second derivatives are found by the Taylor series expansion. Thus $h(\varepsilon) = Q(x + \varepsilon y) = Q(x) + 2\varepsilon Q(x,y) + \varepsilon^2 Q(y) = h(0) + \varepsilon h'(0) + \frac{1}{2}\varepsilon^2 h''(0)$ so that $h'(0) = 2Q(x,y)$ and $h''(0) = 2Q(y)$; similarly (or replacing A by I) we have $g'(0) = 2(x,y)$ and $g'(0) = 2\|x\|^2$. Thus the critical points of $R(x)$ are when $f'(0) = 0$. Letting $x = x_0$ be a critical point with critical value $\lambda_0 = R(x_0)$ we obtain $0 = g(0)h'(0) - h(0)g'(0) = 2\|x_0\|^2 Q(x_0,y) - Q(x_0)(x_0,y) = 2\|x_0\|^2 [Q(x_0,y) - \lambda_0(x_0,y)]$. Since y is arbitrary we obtain, as above, in Theorem 3, that $f'(0) = 0$ or equivalently $Ax_0 = \lambda_0 x_0$ if and only if (λ_0,x_0) is a critical solution

of the Rayleigh quotient.

At a critical solution we have $f(\varepsilon) = f(0) + \frac{1}{2}\varepsilon^2 f''(0) + \ldots$ so that (to second order)

$$R(x_0 + \varepsilon y) \cong R(x_0) + \frac{1}{2}\varepsilon^2 \left[\frac{\|x_0\|^2(2Q(y)) - Q(x_0)(2\|y\|^2)}{\|x_0\|^4} \right].$$

Thus $R(x_0 + \varepsilon y) \cong R(x_0) + \varepsilon^2(Q(y) - \lambda_0\|y\|^2)/\|x_0\|^2 = R(x_0) + \varepsilon^2((A - \lambda_0)y,y)/\|x_0\|^2$. If (x_k, x_k) is an eigensolution of A with $\lambda_1 < \lambda_k < \lambda_n$ then $Q_1(y) = Q(y) - \lambda_k(y,y)$ satisfies $Q_1(x_1) = Q(x_1) - \lambda_k\|x_1\|^2$ $R(x_1)\|x_1\|^2 - \lambda_k\|x_1\|^2 = (\lambda_1-\lambda_k)\|x_1\|^2 < 0$ while $Q_1(x_1) = Q(x_n) - \lambda_k\|x_n\|^2 = (\lambda_n - \lambda_k)\|x_n\|^2 > 0$. Hence for ε small, $(R(x_0 + \varepsilon x_1) < R(x_0) < R(x_0 + \varepsilon x_n)$. The next to last sentence of the theorem about (ℓ,m) saddle points follows by direct computation and the fact that $Q(x_p,x_q) = 0$ if $p \neq q$. Thus if $y = \sum_{m=1}^{\ell} a_m x_m$ then

$$Q_1(y) = Q(y) - \lambda_k(y,y) = \sum_{m=1}^{\ell} [a_m{}^2 Q(x_m) - \lambda_k a_m{}^2\|x_m\|^2]$$

$$= \sum_{m=1}^{\ell} a_m{}^2(\lambda_m - \lambda_k)\|x_m\|^2 < 0.$$

The last statement of the Theorem 4 follows by advanced calculus ideas. The minimum value of R(x) on the unit disc $C = \{x \mid \|x\| = 1\}$ in $\mathbb{R}^n$ is obtained since C is compact and R(x) is continuous. The unit eigenvectors are the only critical points on C so that λ_1 is the minimum value of R(x) on C. Similarly λ_n is the maximum value of R(x) on C. The last sentence of the theorem now follows as R(x) is homogeneous of degree zero.

As an example let $\Lambda = \text{diag}\{-1, 1, 2, 2\}$ with eigenvalues $\lambda_1 = -1$, $\lambda_2 = 1$, $\lambda_3 = 2$ and $\lambda_4 = 2$. Let $\{e_i\}$ be the associated standard eigenvectors where $e_1 = (1, 0, 0, 0)^T$ etc. Then e_2 is a (1,2) saddle point since $\lambda_1 < \lambda_2 < \lambda_3$ and $n - k + 1 = 4 - 2 + 1 = 3$. If $S_1 = \text{span}\{e_1\}$ and $S_2 = \text{span}\{e_3,e_4\}$, then $y_1 \neq 0$ in S_1 and $y_2 \neq 0$ in S_2 implies $R(e_2 + \varepsilon(ae_1)) \cong R(e_2) + \varepsilon^2 a^2 R(e_1) = 1 - \varepsilon^2 a^2$ and $R(e_2 + \varepsilon(be_1 + ce_2)) = R(e_2) + \varepsilon(b^2 + c^2)2$ so that $R(e_2 + \varepsilon(ae_1)) < 1 = R(e_2) < R(e_2 + \varepsilon(be_1 + ce_2))$. As above, if the reader believes our example with a diagonal matrix is too special, since $P^TAP = A$ or $A = PAP^T$, the reader may make up his own matrix A with "diagonal form" A. That is, for any orthogonal matrix O, form $A = O\Lambda O^T$ and $x_k = Oe_k$, the k^{th} column of O.

Section 3. Signature. The purpose of this section is to give an alternate definition of eigenvalues of a real symmetric matrix A or quadratic form $Q(x) = (Ax,x)$. For many problems this definition is more practical than the usual definition of $Ax = \lambda x$. Thus, for example, it is often more practical to think of a zero of a continuous real valued function f(t) as a value such that $f(t_0+)\ f(t_0-)$ is negative. In this example this is not an equivalent definition, but in our case the definitions are equivalent. It also contains the Rayleigh quotient ideas but is easier to apply. Finally this definition involves the signature idea contained above.

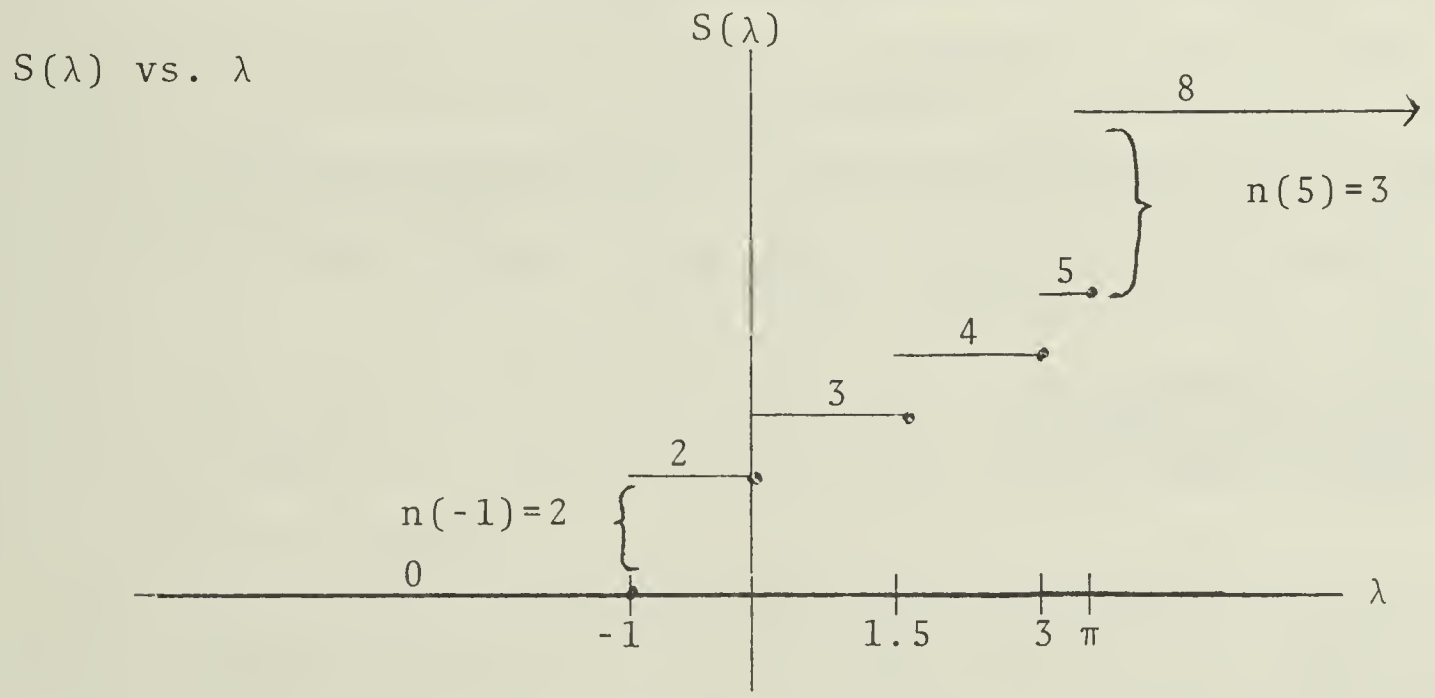
Let A be a real symmetric matrix and $Q(x) = (Ax,x)$ be the associated quadratic form. Let $s(\lambda)$ denote the signature of the quadratic form $J(x;\lambda) = Q(x) - \lambda\|x\|^2$. That is, $s(\lambda)$ is the dimension of a maximal subspace C of IR^n such that $x \neq 0$ in C implies $J(x;\lambda) < 0$. Note that $\lambda_1 < \lambda_2$ implies that $J(x;\lambda_2) - J(x;\lambda_1) = Q(x) - \lambda_2\|x\|^2 - (Q(x)-\lambda_1\|x\|^2) = (\lambda_1 - \lambda_2)\|x\|^2 < 0$ if $x \neq 0$. Thus $J(x;\lambda_2) \leq J(x;\lambda_1)$ and so $x \neq 0$, $J(x;\lambda_1) < 0$, implies $J(x;\lambda_2) < 0$. Thus $s(\lambda_2) \geq s(\lambda_1)$, i.e., $s(\lambda)$ is a nondecreasing, nonnegative, integer valued function. Assume as above that $\lambda_1 \leq \lambda_2 \leq \ldots \leq \lambda_n$ and $x_1, x_2, \ldots, x_n$ are respectively the n eigenvalues and eigenvectors of A or $Q(x) = (Ax,x)$. If $\lambda^* < \lambda_1 = \min R(x)$ for $x \neq 0$, then $Q(x)/\|x\|^2 > \lambda_1$ or $J(x;\lambda^*) = Q(x) - \lambda^*\|x\|^2 > (\lambda_1 - \lambda^*)\|x\|^2 > 0$. That is $J(x;\lambda^*)$ is positive definite and $s(\lambda^*) = 0$. Similarly if $\hat{\lambda} > \lambda_n = \max R(x)$ for $x \neq 0$, then $Q(x)/\|x\|^2 < \lambda_n$ or $J(x;\hat{\lambda}) = Q(x) - \hat{\lambda}\|x\|^2 < (\lambda_n - \hat{\lambda})\|x\|^2 < 0$. Thus $J(x;\hat{\lambda})$ is negative definite and $s(\hat{\lambda}) = n$. The reader may verify as an exercise that the intermediate eigenvalues between λ_1 and λ_n behave as we expect. Thus

THEOREM 5. $s(\hat{\lambda})$ is a nonnegative, nondecreasing, integer value function of λ.

It is continuous from the left, i.e., $s(\lambda_0 - 0) = s(\lambda_0)$ and its jump at $\lambda = \lambda_0$ equal to the number of eigenvalues equal to λ_0, i.e., $s(\lambda + 0) = s(\lambda_0) + n(\lambda_0)$ where $n(\lambda_0)$ is the number of eigenvalues equal to λ_0. Finally $s(\lambda_0) = \sum_{\lambda<\lambda_0} n(\lambda)$.

In future work we will refer to $n(\lambda_0)$ as the nullity of $J(x;\lambda_0)$. In this case it is the dimension of the null space $N(\lambda) = \{x \in IR^n \mid J(x,y;\lambda_0) = 0$ for all y in $IR^n\}$. Note that $N(\lambda) \neq \{0\}$ for exactly n values of λ counting multiplicity. By Theorem 2, this space is the span of the eigenvectors corresponding to the eigenvalues equal to λ_0 since x in $N(\lambda)$ implies $0 = (Ax,y) - \lambda_0 (x,y) = ((A-\lambda_0 I)x,y)$ for all y in IR^n. For future work we note that $m(\lambda) = s(\lambda) + n(\lambda)$ is the dimension of a maximal subspace E of IR^n for which $J(x;\lambda) \leq 0$ on E. This nonincreasing integer valued function is continuous from the right with $m(\lambda_0) - m(\lambda_0 - 0) = n(\lambda_0)$.

We also have an interesting comparison result. Note for $x \neq 0$, $J(x;\lambda) = Q(x) - \lambda\|x\|^2 < 0$ implies $Q(x) < \lambda\|x\|^2$. Thus $s(\lambda)$ gives the dimension of the subspace for which $Q(x)$ is less than $\lambda\|x\|^2$. This concept can be generalized if we replace $\|x\|^2$ by $K(x) = (Bx,x)$ where B is symmetric.



As an example to illustrate signature, if $A = PAP^{\perp}$ where $\Lambda = \text{diag}\{-1, -1, 0, 1.5, e, \pi, \pi, \pi\}$ then the graph of $s(\lambda)$ is given in Figure 4. If $\lambda < -1$ then $J(x;\lambda)$ is positive definite.

Section 4. Lagrange Multipliers. We may also think of eigenvalues as Lagrange multipliers. It is of interest to show that the eigenvalues can be determined solely by a process that maximizes quadratic forms over the unit sphere in IR^n and not on proper subsets of IR^n such as constraints $L(x) = (x,x_i) = 0$ as in the previous work. Once again we assume that $\lambda_1 \leq \lambda_2 \leq \ldots \leq \lambda_n$ are the eigenvalues of A or $Q(x)$ with corresponding orthonormal vectors $x_1, x_2, \ldots, x_n$, respectively. By translation we may assume that $\lambda_1 > 0$, since $A + (|\lambda_n| + 1)I$ has the same eigenvectors as A with eigenvalues translated by $|\lambda_n| + 1$. This assumption is not required in our proof by construction, but avoids some technical difficulties.

We know that $\lambda_n = \max Q(x) = Q(x_n)$ where the maximum is taken over $C = \{\|x\| = 1\}$ the unit ball in IR^n. Notice that $K(x,y) = (x,x_n)(y,x_n)$ is a bilinear form in x and y and $K(x) = K(x,x) = (x,x_n)^2$ is quadratic. We have $K(x + \varepsilon y) = (x + \varepsilon y, x_n)^2 = [(x,x_n) + \varepsilon(y,x_n)]^2 = (x,x_n)^2 + 2\varepsilon(x,x_n)(y,y_n) + \varepsilon^2(y,x_n)^2 = K(x) + 2\varepsilon K(x,y) + \varepsilon^2 K(y)$. Let $Q_1(x) = Q(x) - \lambda_n(x,x_n)^2$. The eigenvalues of $Q_1(x)$ are $\lambda_1 = 0 \leq \lambda_1 \leq \lambda_2 \leq \ldots \leq \lambda_{n-1}$ with corresponding eigenvectors $x_n, x_1, x_2, \ldots, x_{n-1}$. One way of seeing this is that $Q_1(x_i,x_j) = Q(x_i,x_j) - \lambda_n(x_i,x_n)(x_j,x_n) = (\lambda_i x_i, x_j) - \lambda_n(x_i,x_n)(x_j,x_n) = \lambda_i\delta_{in}\delta_{ij}$. This expression is zero if $i \neq j$ or if $i = j = n$. If $i = j \neq n$, then this expression is λ_i. If A were diagonal, i.e., $A = \text{diag}\{\lambda_1, \lambda_2, \ldots, \lambda_n\}$. Then Q_1 would correspond to the real symmetric matrix $B = \text{diag}(\lambda_1, \lambda_2, \lambda_{n-1}, 0)$.

Now $\lambda_{n-1} = \max Q_1(x) = \max Q(x) - \lambda_n(x,x_n)2 = Q_1(x_{n-1})$ where the maximum is over C and not some subset of C. Continuing in this way if $Q_2(x) = Q(x) - \lambda_{n-1}(x,x_{n-1})^2 - \lambda_n(x,x_n)^2$, then $\lambda_3 = \max Q_2(x) = Q_2(x_{n-2})$ where the maximum is over C. Finally note that we may decompose $Q(x)$ into its finite "Fourier series"

$$Q(x,y) = \sum_{k=1}^{n} \lambda_k(x,x_k)(y,x_k).$$

Clearly at each step we have invoked a Lagrange multiplier type rule. This result is stronger than the min-max theory in that we maximize over all of C and do not restrict ourselves to certain subspaces.

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Department of Mathematics
Southern Illinois University
Carbondale, IL 62901

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An Analysis of Successive Spawns of Orange Chromides, *Etroplus Maculatus*

Gary K. Ostrander and Mark S. Lamon
Department of Biological Sciences
Illinois State University
Normal, Illinois 61761

ABSTRACT

Successive spawns of the Orange Chromide, *Etroplus maculatus*, were analyzed for changes in the lipid weight per egg, total weight per egg and total egg number. These factors were used to describe investment in egg production of females through three successive reproductive cycles. There were no signnificant differences between the number of eggs produced or in the average weight per egg from each of the three reproductive cycles. Lipid weight per egg, however, decreased significantly from the first through third cycle. The adaptive significance of the drop in lipid weight is discussed.

INTRODUCTION

Lipids are important for reproduction as indicated in several studies. They contain more than twice as much energy per milligram as carbohydrates or proteins (Hill, 1976). Lipids are used as an energy source during development of many insects. For example Yurkiewicz and Oelsner (1969) examined Indian meal moths, *Plodia interpunctella*, and found that triglycerides decreased during the period from egg laying to egg hatching. Lipsitz and McFarlane (1970) found the same pattern in the house cricket, *Acheta domesticus*. Lipids deposited in the yolk of developing oocytes of the migratory locust, *Locusta migratoria migratoides*, decreased during development (Allais et al., 1964). The importance of lipids in the development of fish has not been examined.

Eggs of the cichlid fish, *Etroplus maculatus* (the orange chromide), were used to examine the significance of lipids in fish eggs. This substrate brooder can be spawned repeatedly after removal of eggs. This may simulate what might occur in nature if a brood was lost. This study examined lipid investment in eggs through three successively forced reproductive cycles.

Orange chromide females spawn repeatedly (approximately three times per month) in the laboratory if the eggs are removed after spawning. There are only two distinct breeding seasons per year in nature; they probably spawn only once

during each season (Ward and Samarakoon, 1981). Eggs removed from successive spawns were analyzed for changes in lipid weight per egg, total weight per egg and total egg number. These factors were used to describe investment in egg production of females through three successive reproductive cycles.

MATERIAL AND METHODS

Orange chromides were imported from Negombo Lagoon, Sri Lanka by Mr. V. Perera, proprietor Lumbini Aquarium, Mt. Lavinia, Sri Lanka. They were placed in 480 litre stock aquaria. Specimens were maintained on a .5/12.0/.5 (simulated dawn/ light/ simulated dusk) light cycle. Aquaria contained deionized water with 1 g marine salt/litre and .5 g commercial baking soda ($NaHCO_3$)/litre as a buffer. Temperature and pH were maintained at 26-28 C and 7.0-8.0 respectively. Light was supplied by overhead 30-watt flourescent bulbs. All experiments were conducted in 75 litre tanks. Photoperiod, salinity, buffering, pH and temperature were identical to conditions in the stock aquaria. Each experimental aquarium contained a slate spawning tent and two plastic plants. Pairs were fed bite size tablets made from ground TetraMin Tablets and methyl cellulose twice daily at 1030 h and after 1300 h.

Four pairs were randomly selected from stock aquaria such that the male was 0.1-1.0 g larger than the female. These pairs remained together until they spawned. The eggs and male parent were removed one day after spawning. The female was transferred on the same day to a second experimental aquarium. A second mate was then introduced. The new pair was allowed to spawn and the procedure was repeated a third time with the same female and a new mate. Eggs were counted after each spawning. They were then freeze dried for 24 h in a lypholyzer (Virtis Research Equipment, Gardiner, New York) and a dry weight determined.

Eggs were divided into 4-6 groups of 35 eggs each depending on the size of the spawn. Each group was ground and suspended in 4 ml of petroleum ether for 24 h in 10 ml centrifuge tubes. Contents of these tubes were resuspended by gentle shaking and then centrifuged for 5-10 min at 2,000 rpm. The supernatant was transferred to pre-weighed 10 ml glass vials by suction pipeting. The precipitate was washed with 3 ml petroleum ether and centrifuged as before. This supernatant was then added to the vials with the first supernatant. The washing process was repeated a second time. The vials were then placed in an exhaust hood where the ether evaporated overnight. Finally, vials were weighed to the nearest ten thousandth of a gram to determine lipid content by weight for each group.

RESULTS AND DISCUSSION

A chi-square analysis was used to examine variance among the groups of each spawn. Linear regression was used to analyze trends of egg number, total weight per egg and lipid weight per egg through successive reproductive cycles. There was no significant differences between either the number of eggs produced or in the average weight per egg from each of the three reproductive cycles. The slopes for egg number and average egg weight plotted per reproductive cycle for a simple linear regression model were not significantly different from zero. Lipid weight per egg, however, decreased from the first cycle through the third. The slope of the regression line was significantly different from zero and negative. The equation

of the line was $y = 1.239 \times 10^{-4} - 1.617 \times 10^{-5} \times$. The coefficient of determination was .7166.

The drop in lipid weight per egg reported above has adaptive significance. Embryos from eggs containing greater amounts of lipid may survive better than embryos from eggs, of the same weight, with less lipid but more carbohydrate or protein. The latter would contain less energy for use by the developing embryo. Blem (1976) suggested high lipid levels may be important in the development of precocial birds. Perhaps high lipid levels in fish allow for development with greater survival. Ward and Samarakoon (1981) suggested that orange chromides spawn only once each breeding season. The evidence reported here supports this hypothesis. Females successively reproducing a second and third time may produce eggs with low survivorship. This would favor the production of just one spawn per breeding season.

Future experiments will follow the same experimental design. However, the survivorship of the offspring will be examined.

ACKNOWLEDGEMENTS

We thank Wayne A. Riddle for use of his laboratory and Jack A. Ward for his criticisms of this manuscript.

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MEMORIAL TO JOHN CHAPMAN FRYE (1912-1982)

H. B. Willman
Illinois State Geological Survey

"This situation has led to a deplorable lack of transfer of information from the geologic community to the political decision makers . . . . . In the next few decades, geologic data and advisory input will be needed more urgently than has ever been the case . . . . . When we view an exponentially increasing population set in apposition to finite mineral and land resources, the urgency of the situation becomes apparent."

In these words John C. Frye expressed concern for the basic responsibility of geology to society, views gained from a long career as head of two state geological surveys, active participation in national organizations, commissions, and panels, and dedication to research in applied and theoretical geology. They were spoken in 1980 at the 75th Anniversary of the Illinois State Geological Survey, where he served as Chief for 20 years (1954-1974). When he died at age 70 on November 12, 1982, at Boulder, Colorado, he had only recently retired after 8 years as Executive Director of the Geological Society of America.

John Chapman Frye was born in Marietta, Ohio, July 25, 1912, the son of Harley Edgar and Maude Vesta (Chapman) Frye. His father was a construction engineer and took part in building the flood-control dams along the Muskingum River in Ohio. John graduated from Marietta College with an A.B. degree in 1934. Marietta College awarded him an honorary Sc.D. in 1955. After a term at Ohio State University, Columbus, Ohio, in 1935, John transferred to the State University of Iowa, Iowa City, Iowa, where, under Professor A. C. Trowbridge, he received an M.S. degree in 1937 and a Ph.D. in 1938. His doctorate thesis, "Additional Studies in the History of Mississippi Valley Drainage," a cooperative study with the Iowa and Illinois Geological Surveys, made for him an early start in the Pleistocene geology of the Midwest, the dominant research activity in his career.

John's professional career after college began with the Groundwater Division of the U.S. Geological Survey in Kansas, where he served as Junior Geologist (1938-1940) and Assistant Geologist (1940-1942). He prepared reports on the groundwater resources of Meade and Atchison Counties and made his first studies of Pleistocene deposits and the Pliocene Ogallala Formation in the Great Plains west of the glaciated region.

In 1942 he accepted an appointment as Assistant State Geologist of Kansas and began a career in administration. By 1943 he was in charge of the Survey. In 1945 he became Executive Director, and in 1952 State Geologist, having served under another eminent geologist, Raymond C. Moore. He also maintained an

academic career as Assistant Professor of Geology at the University of Kansas in 1942, Associate Professor in 1945, and full Professor in 1952, teaching courses in glacial geology and geomorphology.

As Director of the Kansas Geological Survey he expanded studies supporting the mineral industries, particularly those associated with oil, gas, and groundwater, as well as basic research in stratigraphy and paleontology. In his own research he pressed ahead in geomorphology and late Cenozoic stratigraphy, collaborating in several studies with A. Byron Leonard in molluscan paleontology, Ada Swineford in mineralogy, Claude Hibbard in vertebrate paleontology, and James Thorp in soil science. These studies made major contributions to stratigraphy of the loess deposits of central Kansas and to correlation of the nonglacial stratigraphy of central and western Kansas with the glacial sequence in the eastern part of the state. With Moore and others he wrote a major bulletin on the Rock Column of Kansas. With Leonard he wrote a classic volume on the Pleistocene of Kansas. It introduced a classification for Pleistocene sediments based on new stratigraphic data and on principles (then new) that require distinction of units based on rock types from those that represent intervals of time. In 1950, he led the third of a series of field conferences sponsored by State Geologists that brought the new Great Plains stratigraphy to the attention of specialists from many states. In 12 years with the Kansas Geological Survey he was author, or coauthor, of more than 60 papers.

In 1954, John was appointed Chief of the Illinois State Geological Survey succeeding Morris M. Leighton, another eminent Pleistocene geologist who had built the Survey to an outstanding position among state surveys during his 31 years as Chief. It was a time when significant growth of the budget permitted John to develop further growth of staff and expansion of the program. It was also a time of significant advances in technology, particularly geochemistry, geophysics, and computer sciences. New equipment was added in many of the laboratories. A laboratory in isotope geochemistry was established. Neutron activation analysis and low-temperature ashing of coal for mineral-matter studies were started.

Under his administration cooperative research with the Natural History and Water Surveys was greatly increased. With the Natural History Survey a Natural Resources Studies Annex building with additional laboratory facilities and storage for well samples, cores, fossil collections, and other materials was built. In cooperation with the Water Survey a field office was established in Naperville and later in Warrenville to give geological service to the Chicago region.

The many new projects started under his administration included a comprehensive study of the Lake Michigan shoreline features and cooperation with other state agencies on erosion controls. The lake bottom sediments in Lake Michigan were studied and correlated with glacial sediments on the bordering lands.

John encouraged and increased summer employment at the Survey of teachers and students of geology, mostly from colleges and universities in Illinois, which supported active projects and stimulated local interest in Illinois geology.

Within the Survey he increased the joint research projects between the geological and geochemical groups. He restyled the Circular series and introduced new series of publications, called Illinois Mineral Notes and Environmental Geology Notes, designed for rapid dissemination of research. His emphasis on publication stimulated additional research. He was generally accessible to all members of the staff, and he initiated frequent meetings for discussion of problems and programs.

He gained favor from the staff by holding administrative reports to a necessary minimum.

A major influence in increasing the productivity of the Survey was his personal dedication to research, as indicated by the many evenings and weekends he put in at the Survey.

Both of John's successors, Jack A. Simon (1974-1981) and Robert E. Bergstrom (1981-1983) had risen through the Survey organization and had many years experience with him in direction of the organization.

By the time John became Chief of the Illinois State Geological Survey his professional standing and administrative accomplishments were widely recognized, and he was in demand for public service on many national and state committees, commissions, and panels. He served on the National Research Council as a member of the Earth Science Division representing the Society of Economic Paleontologists and Mineralogists (1958-1970), and on many committees, including the Executive Committee of the Earth Science Division (1961-1964, 1972-1974), the Committee on Geologic Aspects of Radioactive Waste Disposal (1956-1964, Chairman 1962-1964), the Advisory Committee on Earth Resources Remote Sensing, advisory to the U.S. Department of Interior (1966-1974, Chairman 1966-1969), the Panel on Geology and Mineral Resources (1966-1971), the Committee on Mineral Science and Technology, advisory to the U.S. Bureau of Mines (1966-1969), and the Committee on Radioactive Waste Management, advisory to the Atomic Energy Commission, including the Panels on Disposal in Salt Mines, on Bedrock Disposal at the Savannah River Plant, and on Engineered Surface Storage (1968-1974, Chairman 1970-1974).

For the U.S. Department of the Interior he was a member of the Secretary's Survey Committee for the Geological Survey (the Van Pelt Committee, 1953-1954) and the Advisory Committee to the Secretary of the Interior for the Geological Survey (1960-1966). He received the Public Service Award from the Department in 1972.

John was elected to membership in the National Academy of Engineering in 1971 and served on the Committee on Environmental Engineering and also the Panel on Solid Waste, advisory to the Department of the Navy.

He served on the U.S. National Commission for UNESCO (1967-1972), on the Executive Committee (1967-1972), the Committee on Natural Sciences (1967-1970), and the Committee on Man and His Environment (Vice-Chairman 1970-1972). He served on the Scientific Manpower Commission (1956-1958), and on the Advisory Committee on Future Oil Prospects of the National Petroleum Council (Chairman for Region 9, 1968-1970).

For the Oak Ridge National Laboratory he served on the Advisory Committee on Health Physics (1959-1972), the Advisory Committee on Chemical Technology (1972-1974), and the Environmental Sciences Advisory Committee (1974). He was a member of the Scientific Committee on Problems of the Environment of the International Council of Scientific Unions.

John served on the American Commission on Stratigraphic Nomenclature (1955-1962), and was Chairman (1957-1959) during the formative stage of the stratigraphic code of 1961. He was on the Commissions on Loess and on Stratigraphy of the International Association for Quaternary Geology (1970-1974).

In his professional services in Illinois, John was a member of the Science Advisory Council to the Governor of Illinois (1964-1974). He held various offices and

committee assignments in the Illinois State Academy of Science and was President in 1962-1963. He served in various offices and was President (1967-1968) of the Illinois Section of the American Institute of Professional Geologists. He was on the Executive Board of the Illinois Mining Institute (1958-1961), the Technical Advisory Committee on Water to the State Department of Business and Economic Development (1961-1967), the Executive Committee of the Illinois Water Center, University of Illinois (1963-1974), the Mining Laws Investigating Commission, State of Illinois (1955-1957), and the Advisory Committee on Environmental Programs, Graduate College, University of Illinois (1969-1974). He also held an appointment as a Professor in the Department of Geology at the University of Illinois (1963-1974).

John was a member of the American Association for the Advancement of Science, the American Association of Petroleum Geologists, the American Geophysical Union, the American Institute of Mining, Metallurgical, and Petroleum Engineers, the Association of American State Geologists (editor 1956-1957, Secretary-Treasurer 1957-1958, Vice-President 1958-1959, President 1960-1961, and Honorary Member 1975-1982), the Geological Society of America (Councilor 1959-1961, Associate Editor 1962-1973, Executive Director 1974-1982), the Society of Economic Geologists, and the Society of Economic Paleontologists and Mineralogists (Vice-President 1965-1966).

When he retired from the Illinois State Geological Survey in 1974 John resigned from most of his public service activities. His new assignment as Executive Director of the Geological Society of America did not lessen his interest in public service, but rather permitted him to give service to geologists through the Geological Society of America.

His transfer from Kansas to Illinois only widened the scope of his personal research interest in the Pleistocene geology of the Midwest. The extensive program on the Pleistocene in Illinois had been based largely on field differentiation and geologic mapping. Under his direction it was expanded to include studies of the clays, carbonates, heavy minerals, radiocarbon dates, textural analyses, weathering characteristics, and mulluscan faunas. He strongly believed in the value of teamwork and, as shown in the accompanying bibliography, coauthored papers on many aspects of the program with George E. Ekblaw, Leon R. Follmer, Herbert D. Glass, John P. Kempton, H. B. Willman, and other of the Survey staff, with part-time Survey associates W. H. Johnson, A. Byron Leonard, and Paul R. Shaffer, and with other colleagues outside the Survey, notably Robert F. Black, Roger B. Morrison, Gerald M. Richmond, and Meyer Rubin.

An early study concerned the origin of the material called "gumbotil," which is widely present on the Illinoian and Kansan till plains and was interpreted as an in-situ soil. John's studies with Ekblaw, Glass, Shaffer, and Willman indicated that the gumbotil consisted of two materials, one a poorly drained, gleyed, in-situ soil, the other a deposit of slow accretion, largely slopewash, that accumulated in shallow depressions. The material cited as typical of gumbotil is largely of the latter origin. The material is not till, and the name "accretion-gley" was introduced for it in 1960.

John's interest in stratigraphic classification continued and resulted in several publications. With Gerald Richmond of the U.S. Geological Survey in 1957 he wrote on soils as stratigraphic units. With Willman and Swann a major change in the policy of the Illinois Geological Survey on stratigraphic classification was in-

troduced in 1958, three years before a similar code was published by the American Commission on Stratigraphic Nomenclature. The new policy used multiple classifications, each classification based on specific characteristics of rocks and each entirely independent. Where only three classifications had been used, it introduced six classifications and later two more were added. Although it carried the practice farther than has been followed by other states, or by the American Commission codes, individual classifications were used by others. The report received commendations from Europeans for presenting the American viewpoint on stratigraphic classification, which, at that time, differed strongly from European practice.

A study with Willman of the loesses along the Illinois and Mississippi Valleys and new radiocarbon dating of shells resulted in a major revision of the classification of the Wisconsinan Stage in 1960.

Although the new policy required significant changes throughout the stratigraphic column, major changes were required in classifying Pleistocene sediments, which had been differentiated somewhat indiscriminately on the basis of genesis and age. The complex glacial, water-laid, and wind-blown deposits were classified for the first time as rock-stratigraphic units — formations and members — based on characteristics similar, at least in principle, to such units in the bedrock. This work was published in a bulletin on the Pleistocene of Illinois with Willman in 1970 and has been widely used.

In addition, John, with Glass and Willman, took part in the study of the mineralogy of glacial tills and their weathering profiles, and with Byron Leonard on Pleistocene molluscan faunas and their environmental significance.

One of John's major interests was in environmental geology, a term coined in 1963 by James E. Hackett of the Illinois Survey. John viewed environmental geology as a means of focusing public and professional interest on the application of geology to the welfare of man. He enlarged on this subject in lectures and publications, and he established a publication series, "Environmental Geology Notes," in the Illinois Geological Survey. Environmental geology has become accepted world-wide as important to the life of all people, and many geological surveys have sections devoted to the subject.

In addition to his research activities at the Illinois Survey, John spent part of his vacations in Texas with Byron Leonard studying the physiographic history of the Red and Pecos Valleys for the Texas Bureau of Economic Geology and in New Mexico working with Leonard and Glass on the Pecos Valley for the New Mexico Bureau of Mines and Mineral Resources. The latter studies established caliche and clay mineral zonation of the Pliocene Ogallala Formation, differentiated the Ogallala from the Pleistocene sediments, and determined the westward limit of the Ogallala. The molluscan faunas indicated the environmental conditions during Ogallala time. John also held an appointment with the Desert Research Institute of the University of Nevada and served as a consultant on studies of Pleistocene sediments.

His unpublished works concern the terraces of the Pecos Valley in New Mexico (with Leonard and Glass), the possibility of glaciation and the origin of the geest in the Driftless Area of Northwestern Illinois (with Willman and Glass), and the stratigraphy of the loess and the Pleistocene history of the Lower Mississippi Valley (with Willman and Glass). Field work on these studies was completed before his death, and reports are in preparation for publication.

John gave to the Survey his very large collection of reprints, which has been

expanded by contributions from others of the staff and grows continuously. It has become particularly useful as a ready source of information on Pleistocene and late Tertiary stratigraphy, and on sedimentation, groundwater, and environmental subjects.

When John retired from the Illinois Geological Survey in 1974, he accepted the appointment as Executive Director of the Geological Society of America and moved to the headquarters at Boulder, Colorado. His appointment was extended two years beyond normal retirement, and after eight years service he retired in June 1982, only five months before his death. John is credited with a dramatic turnaround in the Society's financial health, with major advances in streamlining operations at headquarters, and with leadership in organizing and managing new society activities. The GSA Foundation was established and obtained funding for the Decade of North American Geology, a project to celebrate the Society's 1988 Contennial, for which he provided the initial leadership.

In the announcement of his death from GSA Headquarters, it was stated that "perhaps John's most important contribution to GSA was his ongoing effort to stimulate and encourage greater participation in the Society's affairs by the membership . . . . . The result of that effort has been new awareness among the membership of GSA's position and the role the Society must play in the future to best serve the geological sciences."

Despite the continuing demands of administration and public services, which started early in his career, John attained recognition as an outstanding, broadly informed scientist, and a specialist in the field of late Cenozoic, particularly Pleistocene, geology. He had, if effect, three contemporaneous careers, and he excelled in all because he was remarkably well organized, had an outstanding memory, seemed never to run out of energy, knew nearly everyone, and could write the first draft of a report while others were deciding where to begin.

Field work was his principal relaxation, but he did not neglect his administration responsibilities, often delaying a field trip or returning by air to attend a hastily called meeting. He led many field conferences; two of them, for the International Association for Quaternary Research Congress of 1966 at Boulder, Colorado, permitted him to display Illinois Pleistocene stratigraphy to geologists from many countries and resulted in a continuing exchange of ideas on intercontinental correlations.

John was particularly efficient in the field. Nearly always the first in the party to get his well-worn trench pick into the ground, he did more than his share of the physical work. He drilled an auger hole faster than most, and when it was particularly hard going could always make a few more inches, so he did most of the augering. He insisted on making adequate notes and sketches before leaving an exposure. Before turning in every night he called for a conference and then wrote a summary of the findings of that day.

John was not an early starter, but he used the day to the last glimmer of light, even when two hundred miles from home. Field time was precious, and he drove from locality to locality at the maximum rate car and highway could stand. Your first ride with him on a gravel road was a chilling experience, but you soon realized that he was a skillful driver, and survival was most likely. Oddly enough his most severe accident, although minor, occurred in a town when the car was standing still, waiting for a rushing fire truck to pass. Unfortunately, the driver of the car

coming behind had eyes only for the fire truck. John suffered the only injury, a very sore neck from the whiplash.

Only his close associates realized that John was somewhat timid about meeting people, a characteristic concealed by his friendly manner and his effective presentation of talks at meetings. He had a strong aversion to anything pretentious or self-promoting. Public plaudits were not his style. He appreciated and rewarded accomplishments and was generous in sharing the prestige of senior authorship. When he arrived as Chief of the Illinois Geological Survey, formality went out the door, to the consternation of some, the pleasure of many.

Dr. A. Byron Leonard, his close associate for many years, wrote an appreciative memorial of John for the Kansas Geological Survey entitled "A Better Friend Hath No Man," which expresses a deep regard that was clearly mutual, and was shared by many. His friends extend from coast to coast and to many countries.

John was a devotee of classical music. His principal hobby was photography, and he built equipment for his darkroom. He was attached, spiritually, to his cars, and, in his early days at the Survey, it was easy to believe that nothing could be seriously wrong with a man who was so devoted to an old Studebaker station wagon. John was a Mason, Rotarian, and a member of Sigma Xi, Sigma Gamma Epsilon, Alpha Sigma Phi, and the Cosmos Club of Washington, DC.

He and his wife, Ruth, were frequent and gracious entertainers, and an evening in their home was always a pleasant time with stimulating exchanges of ideas on current events as well as those of eons past.

After he retired from the Illinois Geological Survey John returned each summer, except his last, to continue field studies in Illinois. It was a particularly enjoyable time when Ruth accompanied him. John's intense dedication to his professional career came not only from the challenge of his opportunities but even more from strong devotion to his family.

John married Ruth L. Heizer, of Westerville, Ohio, his girlfriend of high school and college days in Marietta, in 1936. They had two daughters, Sally Jean Schwartzlose, of Evanston, Illinois, and Terri Ruth Toedter, of Riyadh, Saudi Arabia, and three grandchildren, all of whom survive. A son, John Douglas Frye, died in 1970.

In August of 1982 John enjoyed his usual field work in New Mexico with Byron Leonard and members of the New Mexico Bureau of Mines and Mineral Resources, and he seemed to be in normal good health. He was planning on going to the Geological Society of America meeting in early November, but he became ill only a few days before scheduled departure. An operation for all stones revealed an advanced stage of cancer. He lived only about two weeks afterward. His family was with him when he died at Boulder on November 12, 1982.

We wish that John could have had time for another career — retirement. He probably would have made a big project of it, with him doing most of the work. John certainly enjoyed his life. It was full enough for any man.

Many members of the staff of the Illinois State Geological Survey contributed to his memorial. Other memorials for John C. Frye include the following: William C. Ackermann, National Academy of Engineering, in press; Robert E. Bergstrom, State Geologists Journal, v. XXXXV, p. 54-55, 1983; and A. Byron Leonard, The Journal, Kansas Geological Survey, v. 5, no. 1, p. 6-8, 1983.

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Toxicities of Natural Foods in Man: A Survey

Harold Kaplan
Department of Physiology
School of Medicine, Southern Illinois University
Carbondale, Illinois

Foods are considered to be substances that nourish cells and enable them to grow, multiply and repair themselves. Many foods may produce bodily harm, however, by some intrinsic toxicity or by excess consumption, contamination, preparation, inherited inability to process them, hypersensitivity of the individual, or other.

The literature is scattered and voluminous and the early reports are untrustworthy. In the present discussion, man rather than animals is emphasized as well as food toxicity encountered chiefly in the United States. We have not included any commentary on federal policies of increasing toleration of food practices dangerously leading to toxicities (see Marshall, 1982).

FOODS CONTAINING CARCINOGENS

Saccharin

Saccharin is the most widely used sugar substitute. In large doses it incites bladder tumors in laboratory animals and the FDA has placed it on a regulated list.

Tea

The black teas from Ceylon and India contain considerable *tannin* and *gallic acid*. These are controversially suspect as causative factors in esophageal cancer (Lewis and Elvin-Lewis, 1977, p. 119).

CONTAMINANTS FAVORING CARCINOGENICITY

Fungus Contaminants (Mycotoxins)

Some strains of Aspergillus, a mold, produce *aflatoxins*. The one found most often in food is aflatoxin B_1. It inhibits protein synthesis, causing the death of liver cells particularly. B_1 is one of the most potent hepatocarcinogens known. It is found chiefly in figs, peanuts and other nuts, and cereals (Osborne, 1982).

There is a toxic substance called *patulin* which can occur in apple juice due to contamination by molds. The substance is suspected of being carcinogenic (Crosby, 1979).

Toxins can be present in food that is not visibly moldy and they can persist even if the food is cooked or otherwise processed.

Polycyclic Aromatic Hydrocarbons (PCHs)

PCHs are an important class of carcinogens because they are being widely dispersed in the atmosphere, soil and water from the smoke and smog of industrial cities and some have tumorigenic potency. Dangerous levels of PCHs are now detected in many foods, particularly leafy vegetables (Harvey, 1982).

CONTAMINATION OF DRINKING WATER

Water is a food. Contamination by industrial wastes possibly leading to carcinogenicity is a huge problem and will not be considered here. There is atmospheric contamination of an incidental nature, however, nitrates posing a problem. It is contended that a high nitrate concentration in water supplies provides a risk of gastric cancer (Walters and Smith, 1981).

A concern about nitrates in water peripheral to the cancer issue centers on their reduction to nitrates by bacteria which can live in an infant's stomach due to low acidity therein. Hemoglobin, especially the persisting fetal type in the infant, is susceptible to oxidation by nitrites and methemeglobin is formed. Oxygen delivery is impaired. Cyanosis in infants who are given water from wells is a manifestation of nitrate toxicity (Gleason et al., 1969, p. 100).

Water per se can produce ill effects. Massive overdrinking (which has been used in torture) produces the syndrome of "water intoxication". Cerebral edema, convulsions and coma can follow.

Water causes gastric distress in many persons because it can excessively stimulate gastric juice, which irritates the stomach lining. That is why "club soda", which contains sodium bicarbonate, may produce no such symptoms.

FOODS DEVELOPING CARCINOGENIC PROPERTIES BECAUSE OF PREPARATION

Charcoal broiling can result in the formation of carcinogenic hydrocarbons. Smoke imparts a flavor derived from phenolic materials produced when wood is burned in conditions of oxygen deficiency. While the phenolic flavors are formed, polynuclear aromatic hydrocarbons are also produced. Some of these, especially *3, 4-benzpyrene* and *benzanthracene*, are carcinogenic. These occur in smoked fish, caramelized sugar and roasted coffee (Crosby, 1979). Benzpyrene is the ubiquitous carcinogen in city smog. Its metabolites, particularly *epoxides*, are active in producing malignancy in animals and possibly in man.

Fats and oils subjected to prolonged overheating by frying are controversially thought to produce carcinogens. The toxicity is claimed to increase if an oil is used repeatedly and if a shallow pan allowing oxidation is used.

Yannai (1980) presents a succinct review of toxicity induced by food processing. Thus, a cured meat such as bacon contains trace amounts of *N-nitrosodimethylamine* (NDMA) and on frying bacon N-nitrosopyrrolidine is formed. Both substances are claimed to be carcinogenic (Gough et al., 1976). Fortunately, both nitrosamines get lost to a considerable degree in the vapor during cooking, although some remain in the rasher and in the cooked-out fat (Holmes and Woodburn, 1981). Wasserman et al. (1978) state that frying bacon at low or medium

heat for less than 10 minutes can result in the least nitrosamine levels, although Pensabene et al. (1974) report that N-nitrosamine formation is dependent on temperature and not time.

The nitrite in bacon processing imparts flavor and color and prevents growth of *Clostridium botulinum*. Since the nitrites react with amines in the body to produce nitrosamines which are inconclusively implicated in cancer of laboratory animals (Pierson and Smoot, 1982), there are proposed substitutes or sparing agents (Widdus and Busta, 1982).

In 1978 it became known that NDMA was present in domestic and imported beers and that barley malt was the source (Havery et al., 1981). FDA regulations to modify the malting process have reduced the present nitrosamine levels markedly.

FOODS CONTAINING MUTAGENS

Mutagens are agents that produce genetic mutations and they have a wide distribution in the diet. There are *mutagenic flavonoids* in many vegetables and mutagenic activity is said to exist in extracts from coffee and tea (Maugh, 1982). There are controversial opinions as to whether mutagens in foods contribute markedly to the risk of cancer (Committee, 1982).

A. Caffeine

Although there is no evidence that caffeine is involved in carcinogenesis, there is a claim that it induces chromatid breakage (genetic damage) in human cells *in vitro* and that it could prove to be a dangerous mutagen (Kahlmann et al., 1968).

B. Charring of Meat and Fish

Charred meat and fish can develop mutagenic potential. Tryptophan, serine and glutamic acid may produce mutagens at high temperature pyrolysis. Difficult decisions arise. Although cooking of meat and fish may produce mutagens, it also destroys pathogenic organisms. Further, some vegetables that contain mutagens have a high nutritive value.

FOODS CONTAINING TOXICANTS (MAINLY CYANOGENIC GLYCOSIDES)

A. Fruits

Apple (*Malus* spp.)

There is a toxic part of apples, mainly in the seeds. Lewis and Elvin-Lewis (1977) cite a case where one cup of seeds was eaten and caused death, from cyanide poisoning.

The amino acid phenylalamine in the seed converts to the cyanogenic glycoside *amygdalin*. This hydrolyzes on digestion to hydrocyanic acid. The cyanide poisons oxygen carriage by cytochrome oxidase. Victims may die within minutes of exposure. The lesson here is (1) that all parts of a food are not harmless, and (2) that children, especially, must learn this early.

Apricot (*Prunus armeniaca*)

Apricot plants contain amygdalin in the seeds, bark and leaves and ingestion should be avoided (Lewis and Elvin-Lewis, 1977, p. 41). Laetrile, legal in Mexico as a cancer cure, is an extract of apricot pits. The theory is that the extract yields

cyanide which preferentially attacks tumor cells. Cyanide is released by beta-glucuronidase common to tumor cells.

Elderberry (*Sambucus* spp.)

The fresh leaves, stems and roots contain a poisonous alkaloid, and a cyanogenic glycoside called *sambunigrin* causing cyanide poisoning. Red elderberries are very toxic.

The fully ripe fruit is harmless and is used in pies, wine, jelly and pancakes. Other parts of the plant are used as drugs.

Children using peashooters made of elderberry stems have been poisoned (Lewis and Elvin-Lewis, 1977, p. 50).

Cherries (*Prunus* spp.)

Seeds and leaves contain the cyanogenic glycoside *amygdalin*. The seeds are the most toxic. The fleshly part of sweet and of sour cherries is edible. That of the laurel cherry (*P. laurocerasus*) contains amygdalin.

Pear (*Pyrus communis*)

Pear seeds contain amygdalin and are potential poisons.

Peach (*Prunus persica*)

All parts of the peach contain amygdalin, concentrated mainly in the bark, leaves and seeds. Poisoning has followed ingestion of peach kernels (Liener, 1980, p. 152).

Plum (common is *Prunus domestica*; wild is *P. americana*)

The plum is the least toxic member of the genus Prunus, but the leaves and seeds contain cyanogenic glycosides that convert to hydrocyanic acid.

Prune (*Prunus domestica*)

The prune is the partially dried fruit of a plum. The laxative effect of prunes is well known. Prunes contain derivatives of *hydroxyphenylisatin* which acts as a gastrointestinal irritant.

Almond (*Prunus amygdalus*)

Almonds are the fruit of the almond tree. The seeds of the sweet varity are edible. It is difficult to separate the edible sweet almond from the bitter almond (*Prunus amygdalus* var. *amara*). The seeds of the bitter plant contain high yields of amygdalin (Abrol, 1967). Lewis and Elvin-Lewis (1977, p.41) state that 50 to 70 bitter almond nuts are fatal for adults and 7 to 10 for children.

FOODS CONTAINING TOXICANTS

B. Vegetables

Onion (Allium cepa)

A toxic principle thought to be an alkaloid may cause an anemia in man if onions are eaten in "large" amounts for one or more weeks (Schmutz et al., 1979, p. 155). The bulb contains the toxic material. Other related suspected foods are chives, garlic, leeks and shallots.

Onions and other plants in the genus Allium contain sulfur compounds called *glucosinolates*. These give rise to thiocyanates, isothyocyanates, nitriles, goitrin and progoitrin. Glucosinolates substitute for or displace iodine, preventing its uptake or accumulation in the thyroid gland. Heat reduces the direct action of these substances. Normal patterns of consumption cause no harm.

Eggplant (Solanum melongena)

Eggplant is a fruit eaten as a vegetable. Botanically, it is a berry. Its leaves, stems and fruit contain solanaceous alkaloids that are claimed, without solid evidence, to be poisonous. The plant belongs to a nightshade family long suspected as being potentially toxic. Glycosides of *solasodine* are common in eggplant and solasodine is teratogenic in hamster fetuses (Kinghorn, 1979, p. 75).

Fava Bean (Vicia faba)

Fava (broad) beans may produce acute febrile hemolytic anemia. The condition is called favism. Crises due to dry or cooked beans are of moderate intensity but severe attacks follow ingestion of fresh, raw fava seeds.

Sensitivity is hereditary and involves a deficiency of glucose-6-phosphate dehydrogenase. Causative toxic substances may be *divicine* and *isouramil* (Liener, 1980, p. 283).

The plant is widely cultivated in parts of the United States. Most cases result from eating fresh beans and thus peak incidence occurs at harvest.

Lima Beans (*Phaseolus lunatus*)

Lima beans contain a lectin (protein hemagglutinin) called *phaseolunatin* or *linamarin* (Lewis and Elvin-Lewis, 1977, p. 44). This hydrolyzes in part to hydrocyanic acid. Thorough cooking reduces the danger of poisoning, but may not fully destroy the toxic principle. Preliminary soaking before cooking helps eliminate the toxic lectin.

Kidney (Navy) Beans (*Phaseolus vulgaris*)

The kidney bean contains lectins (hemagglutinins) associated with human gastrointestinal disturbances. The toxin is completely destroyed by boiling (Bender and Reaidi, 1982). Of interest, the bean also contains inhibitors of alpha-amylase from the saliva and pancreas. The active inhibitor, called *phaseolamin*, when taken orally blocks starch digestion and this is the principle behind starch-blockers for weight control (Bo-Linn et al., 1982). The FDA is now in litigation as to whether these agents are drugs or foods.

Wheat (*Triticum aestivum*)

Wheat also contains the alpha-amylase inhibitor. This may persist through bread baking and infants or adults having impaired digestive enzymes may not destroy the inhibitor. A similar problem also arises in some wheat-based breakfast cereals (Liener, 1980, p. 450).

Bread cannot be made without the gluten of wheat and some uncertain fraction in the gluten exacerbates the symptoms of patients with celiac disease (Goodhart and Shils, 1973, p. 423). Wheat is aso a very common food allergen, due in part to albumin and gliadin.

Spinach (*Spinacia oleracea*)

The leaves of spinach contain salts of *oxalic acid* that could become precursors of calcium oxalate crystals. Excesses have been controversially claimed to cause precipitates in the kidney. Spinach also lowers blood sugar (Lewis and Elvin-Lewis, 1977, p. 218). The concentrations of oxalates or hypoglycemic factors are not ordinarily toxic.

Between 1959 and 1965 in Germany, 15 cases of *nitrite* poisoning followed ingestion of spinach by babies (Simon, 1966). Poisoning occurs only if spinach contains much nitrate, which bacteria of the stomach convert to nitrite. Infants less than three months of age should not be given spinach because of bacterial action.

Arena (1979, p. 718) cites the death of a five-year old girl who ate a large number of raw spinach leaves. Epstein (1969) states that vegetables in highly fertilized soil contain 1,000 to 5,000 ppm of sodium nitrate, well exceeding the maximum permissible amount in foods.

Nitrates can convert to nitrite in cans and the nitrite reacts with the metal to produce nitrogen which swells the can. Metal cans have caused toxicity for other reasons, essentially because of dissolved tin or the lead solder used and, for that matter, even plastic containers contain toxic polymers (Liener, 1980, p. 417).

Potato (*Solanum cuberosum*)

The "Irish" potato is edible so long as it is not very green. Since it is a modified stem, a potato exposed to light for a period of time becomes green and it may accumulate toxic amounts of a glycoside alkaloid called *solanine*. The toxic parts of the potato include green areas under the potato skin and the sprouts. The skin is harmless when eaten in normal quantities. A concentration of 20 mg solanine/100g fresh weight is thought to be the maximum safe level for market potatoes, to prevent gastrointestinal and neurologic disorders. Fatalities are rare because sprouted potatoes are peeled. Also, solanine in boiling is leached out into the water (Koelling, 1982), although cooking does not destroy it. Solanine is a cholinesterase inhibitor.

Rhubarb (*Rheum rhaponticum*)

The leaves and edible stalks of rhubarb contains rather high quantities of oxalic acid. This was once thought to be the cause of vomiting and enlargement of the liver that follows ingestion of raw leaves and stems. It is much more likely that the toxicity is due to *anthraquinone glycosides*. These are present in leaves, especially in early summer. About 10 to 20 grams of fresh leaves are highly irritating (Fairbairn, 1976, p.15). Death has sometimes followed ingestion of rhubarb (Leiner, 1980, p. 453).

Mushroom (*Amanita* spp)

Mushrooms are unpredictable. Some species are only occasionally poisonous. Some are poisonous only if eaten raw; others if eaten in certain seasons or stages of maturity. Cooking does not destroy all the toxins.

Some people become ill if they drink alcoholic beverages when eating mushrooms. Use of certain drugs may make a harmless mushroom toxic for some people.

The most common poisoning in the United States is from *Amanita verna* and *A. brunnescens*. Poisoning by these and the more rare *A. phalloides* is due to thermostable toxins that destroy cells. Toxins are cyclopeptides which include *phalloin*, *phalloidin*, and *amanitins*. Poisoning by *A. muscaria* is due to *ibotenic acid* and *muscimol* (Kinghorn, 1979, p. 25). There are other mushrooms that contain *psilocybin* and *psilocin* which produce hallucinations. Additional toxicants have been extracted from still other species.

Yams (*Dioscorea* spp.)

Yams are among compounds suspected of causing teratogenesis in man and other animals (Lewis and Elvin-Lewis, 1977, p. 95). Certain yams (*D. hirsuta*) contain an alkaloid, *dioscorine*, which produces paralysis of the central nervous system (Glasby, 1975, p. 490).

Sweet Potato (*Ipomoea batatas*)

Sweet potatoes produce metabolites called *phytoalexins* in response to stress (Salunkhe and Wu, 1977). An abundant metabolite is *ipomeamarone*. This is a

hepatotoxin in mice and a suspected toxin in man. It is not eliminated by baking or boiling and it is best to avoid potatoes with blemishes or discolorations.

C. Food Colors; Spices

Nearly 65 synthetic colors are known to be in use around the world. Khanna and his co-workers (1980) review these substances and they deplore the fact that some appear to be highly toxic and nevertheless are insufficiently regulated. In the United States there are about eight color additives and the FDA certifies them.

Monosodium Glutamate

This is the responsible agent for the digestive upset known as the "Chinese restaurant syndrome." It is especially dangerous in baby foods because of reported damage to the brain in newborn animals (Grollman and Grollman, 1970).

Hot Peppers (*Capsicum* spp.)

Red chili peppers (*C. annuum*) can irritate the entire gastrointestinal tract. The symptoms are more severe with the tobacco pepper (*C. frutescens*) which is considered to be a hepatocarcinogen in rats.

Nutmeg (*Myristica fragrans*)

Nutmeg is the ripe seed of *M. fragans*. It is a powerful hallucinogen. This is due to a combination of volatile oils, *myristicin*, *elemicin* and the weak carcinogen *safrole*. Ingestion of large quantities of nutmeg causes stupor and death (Merck Index, 1960, p. 700).

Sage (*Salvia officinalis*)

Sage represents the dried leaves of the plant. Large doses may cause nausea, vomiting and constipation (Merck Index, 1960, p. 918).

D. Milk

Bovine milk is the main source of *orotic acid* in the human diet. Levels in cow's milk increase from 21 to 84 ppm from days 2 to 45 of lactation. Because only 10 ppm may affect liver metabolism and cause liver necrosis in rats, there is need to evaluate its possible toxicity in milk powders used in humans (Okonkwo and Kinsella, 1969). Robinson (1980) does not think that orotic acid poses a serious problem in man, but believes that research is needed on this point.

FOODS CONTAMINATED BY TOXICANTS

Milk

There is considerable concern about the level of pesticide residues in both bovine and human milk. Dillon et al. (1981) present a survey of this problem. Lee and Lorenz (1979) note other hazards of milk, i.e., in the cardiac-prone hypercholesterolemic person, in the lactose-intolerant person, and in the allergic person. Infants with hereditary disturbance of galactose metabolism develop serious symptoms with ingestion of milk. Such galactosemia demands rigid exclusion of lactose and galactose from the diet. Speckmann et al. (1981) claim that intolerance to lactose in an 8-ounce serving of milk is rare.

Rye (*Secale cereale*)

Ergot is the toxic chemical constituent of the sclerotium (branching filaments; hyphae) of *Claviceps purpurea*, a fungus that parasitizes kernels of rye (and wheat) chiefly. Ergot contains *ergonovine*, an alkaloid causing human ergotism. This is

highly controlled today, but it killed millions of people in the Middle Ages. There are burning sensations and gangrene in the extremities, and also abortion and convulsions. Epidemics in the Middle Ages were referred to as "St. Anthony's Fire" because gangrenous limbs looked as though they were burned.

Fish and Shellfish

Fish muscle contains histidine which can form histamine by bacterial action if left standing. Canned tuna fish has been a source of histamine poisoning (Rice et al., 1976), and the disorder called scombroid poisoning is caused chiefly by histamine.

Shellfish such as clams eat the dinoflagellates *Gonyaulax catenella* or *G. tamarensis* and thereby acquire a nitrogenous compound called *tetrodotoxin* which does not harm the shellfish, but which is often a fatal neurotoxin to man. At certain times of year the dinoflagellates so multiply as to produce a dangerous "red tide" poisonous to clams, crabs, oysters, shrimp and other marine organisms. A neurotoxin is involved called *saxitoxin* which is highly poisonous when man ingests the shellfish and this toxin is not destroyed by cooking. Fish eggs can also contain toxicants (see Liener, 1974).

Meat Grilled on Wooden Skewers

Wooden skewers used to hold "hot dogs" and other meat for grilling can introduce hazards. There are skewers made from the stems of an evergreen, *Nerium oleander*, which contain cardioactive glycosides that have caused deaths (Liener, 1974). The poisons are *oleandroside* and *nerioside*.

Beer Treated with Cobalt

At one time cobalt was added to beer to stabilize the foaming qualities. It took many years before cobalt was incriminated as the cause of severe heart attacks in heavy beer drinkers and this additive was discontinued (Alexander, 1969).

FOODS ACTING AS TOXICANTS BECAUSE OF INHERITED DEFECTS IN THE INDIVIDUAL

For a person having severe pancreatic diabetes, where from birth insulin function may be deranged, a single ordinary meal containing cane sugar (sucrose) may set off a train of metabolic events leading to disastrous results.

Fructose occurs in many fruits and is the predominant sugar in honey. Some people are born without an enzyme, *aldolase B*, and for them fruits (and also cane sugar) have to be completely excluded to prevent gastrointestinal pain, vomiting and hypoglycemia (Cox et al., 1982). The disorder is acquired from a double dose of a recessive gene.

Persons who by gene defect lack the enzyme *lactase* fail to break down milk sugar (lactose) and they may not tolerate milk.

Fatty foods, especially french fried potatoes and onions, are anathema to most people who suffer by inheritance or otherwise from disorders of the biliary tract. This is expected since many nutrients affect the quality and amount of the bile, the movements of the gallbladder, and the tone of the sphincter of Oddi.

These so-called inborn errors of metabolism also include protein disturbances. In phenylketonuria, there is a deficiency of *phenylalanine hydroxylase* and phenylalanine intake must be sharply reduced by feeding artificial protein substitutes. In "maple syrup urine disease," the infant must ingest an artificial formula free of

valine, leucine and isoleucine. These are only examples of a class of amino acid toxicities.

FOODS AS VITAMIN ANTAGONISTS

A. Biotin Antagonist — Raw Eggs

Raw eggs contain a glycoprotein called *avidin*, present in the egg white up to 0.05% of the total protein content. One molecule of avidin binds four molecules of biotin (vitamin "H"). This deprives the person ingesting raw eggs of an essential growth factor (Comments, 1982-83).

B. Thiamin Antagonists — Seafoods, Vegetables, Fruits

Fish such as carp, herring, swordfish and lake trout contain thiamin antagonists (Goodhart and Shils, 1973, p. 415). There is also anti-thiamin activity in blackberries, black currants, red beets, Brussels sprouts, red cabbage and spinach. There are antagonists for niacin in corn and millet, for pantothenic acid in yeast and for riboflavin in the akee plum.

C. Multivitamin/Mineral Preparations

Multivitamin/mineral preparations contain analogues for B_{12} which are claimed to be harmful to human vitamin B_{12} metabolism (Herbert et al., 1982). It seems advisable to omit B_{12} from vitamin/mineral preparations.

Vitamin Poisoning — Hypervitaminosis

Only those vitamins are considered herein whose known or probable toxicities have strong documentation. Gilman (1980, p. 1553) lists recommended daily vitamin allowances for the reference man, woman and child. Quantitative values for toxic thresholds are controversial. Poisoning does not occur with ordinary daily consumption.

The recommended adult allowance for vitamin A is 5,000 I.U. Poisoning occurs after ingesting up to 100,000 units (30 mg) daily for several months. Food faddists may ingest toxic levels. Infants need about 1,500 I.U. and doses of 18,500 daily for 1 to 3 months are reported to be toxic (Goodhart and Shils, 1973, p. 153).

For vitamin D, the daily requirement for infants and adults is 400 I.U. There is a danger in giving supplements such as 4,000 I.U. in fortified foods to infants (Goodhart and Shils, 1973, p. 160). Ingestion of 50,000 units daily to children and adults should be avoided for any long periods of time.

For other vitamins, toxicities are either unknown or they occur at such repetitively high values that they may be considered relatively safe. The reports are conflicting.

GOITROGENIC FOODS

Soybean (*Glycine max*)

Goiters have occurred in infants fed soy milk or soy flour (Hydovitz, 1960; van Wyk et al., 1969). There are also many goitrogenic foods in the genus Allium, but it is only excess consumption that may be associated with toxicity.

Soybeans are toxic in other regards. One possible toxic factor, which is common to other legumes, is a *trypsin inhibitor*. Trypsin is essential in the small bowel for protein digestion. The inhibitor, present in the raw bean, is inactivated by cooking.

Soybeans are also hemagglutinins (phytoagglutinins or lectins) and this may be partly responsible for the gastrointestinal disturbances if raw soybeans are eaten.

A valid explanation is still needed to explain the growth-inhibiting action in raw soybeans (Goodhart and Shils, 1973, p. 418).

TOXICITY OF FOOD ADDITIVES

Preservatives and Sweeteners are Emphasized Below.

Preservatives

These substances extend the shelf life of foods. Shtenberg and Ignat'ev (1970) urge that sulfites and benzoates should be restricted, substituting sorbic acid. Sorbic acid and potassium sorbate are considered safe by the FDA.

Sodium nicotinate, a fresh meat color extender, has caused food poisoning, expressed particularly in edema of the extremities. This additive is not legalized in some states.

Sodium nitrite is used to prevent growth of *Clostridium botulinum* and to preserve the color of meat and fish in salting and pickling. Nitrites are potentially poisonous in several ways, one involving methemeglobin formation. Another is in formation of N-nitrosamines, formed either in processing foods or in the digestive tract by reaction with amino compounds. By similar mechanisms, nitrites react with phenolic compounds, forming aromatic nitroso substances, some of these nitrophenols being suspected of carcinogenicity (Challis, 1973).

Sweeteners

There are some toxic synthetic sweeteners. Cyclamates were banned in 1969 because they produced bladder cancer in rats. Saccharin is now a regulated substance with suspected carcinogenicity. Crosby (1976) reviews several sweetening agents.

Cane sugar as a natural sweetener is in a sense an additive and its local dental effects are of interest. Meals high in cane sugar favor colonization of cariogenic organisms on teeth. Restriction of cane sugar reduces bacteria such as *Streptococcus mutans* in dental plaques (Newbrun, 1982). Bibby and Mundorff (1975) blame acid candies, beverages and fruits for the highest dissolution of enamel.

POISONING BY EXCESS MINERAL INGESTION

A. Sodium Chloride (Table Salt)

In the United States, the daily sodium intake per person is three or more grams. An estimated safe and adequate daily intake should range from 1.1 to 3.3 grams (48 to 143 mEq.) (National Research Council, 1980). A moderate restriction is one gram. The hypertensive influence of table salt is firmly documented and the FDA proposes labelling the sodium content of processed foods.

Salt poisoning has occurred in hospitalized infants where salt was mistaken for cane sugar. Renal damage and convulsions followed. In the neonate, the repeated intake of skim milk has produced salt intoxication.

B. Iron

The daily iron requirement is 10 to 20 mg in adult males and 8 to 15 in females (Gilman et al., 1980). Finch (1982) states that a normal person can control the accumulation of body iron despite ingestion of 5 to 10 times the normal amount. Other writers note that excesses can be dangerous because excretion hardly increases, so that iron deposits in vital organs.

Some wines of southern France contain 16 mg of iron per liter and even

American wines supply several mg daily. The Bantus of South Africa brew a beer in iron kettles and the beer contains more than 40 mg of iron per liter. A Bantu male often consumes 100 mg of iron per day in beer (Emery, 1982). A disease called secondary hemochromatosis, often fatal, occurs.

The Food and Nutrition Board of the National Academy of Sciences recommends a daily allowance of 10 mg daily for children in the first six months to supplement milk. This may be too high. Also, it is inadvisable for an adult to take vitamin/mineral preparations containing iron if iron is being ingested sufficiently from other sources.

C. Calcium

The minimal adult requirement is 800 mg per day (Gilman, 1980, p. 1553). The predominant source is dairy products. Healthy serum contains 10 mg/dl. Levels exceeding 14 are dangerous and 18 mg/dl may cause death. Ingestion of daily quantities of calcium salts is ordinarily unlikely to cause toxicity. The so-called "milk-akali syndrome" can be exacerbated, however, by ingestion of large quantities of milk.

D. Fluoride

Man obtains fluoride from ingesting plants and water. Incidental sources include food additives, e.g., baking powder. Combinations of fluoridated water, toothpastes and certain foods could conceivably produce a problem especially in children. This is a developmental problem in teeth where 1.7 ppm of fluoride in water produces mottled enamel before the teeth have erupted (Gilman, 1980, p. 1547). At 4 to 6 ppm, mottling approaches 100%. Severe mottling is associated with disorders in bone mineralization. Fluoridated drinking water is restricted to 1 ppm optimally.

E. Selenium

The estimated safe daily intake of selenium is 0.05 to 0.02 mg (Gilman, 1980, p. 1555). Where the soil and vegetation have a high selenium content, livestock develop "alkali disease" or "blind staggers." The human population in such areas is claimed, controversially, to develop gastrointestinal symptoms and liver disease from ingestion of meat, eggs, milk and vegetables (Goodhart and Shils, 1973, p. 393; Salunkhe and Wu, 1977).

FOODS INDUCING MINERAL DEFICIENCIES

There is a large literature on *phytates* as toxins. These commonly occur in food plants and they interfere with the intestinal absorption of essential minerals by forming insoluble complexes with them. This produces the hazard of mineral deficiency in parts of the world that rely on cereal proteins (Reddy et al., 1982).

FOODS AFFECTING CARDIOVASCULAR FUNCTION

The literature is voluminous concerning the development of atherosclerosis as influenced by dietary excess of cholesterol and low density lipids, but these will not be considered further here.

A. The Effects of Coffee

The cardiac effects of coffee are highly controversial. Lewis and Elvin-Lewis (1977, p. 181) cite data to the effect that the risk of developing myocardial infarc-

tion is about twice as great for heavy coffee drinkers than for non-drinkers. This does not imply that caffeine is the cause since tea does not produce the same effect.

B. Orange Oil

Orange oil is expressed from the fresh peel of the ripe fruit of the orange. *Citral* is a constituent of this oil and is thus present in marmalade, fruit juices flavored with the oil, and orange drinks made by compressing the whole fruit. There is a question whether high consumption of such foods might damage blood vessels, as seen in experimental animals (Liener, 1980, p. 442). Citral is found to greater extent in oil of lemon, which is a flavoring agent. Goodhart and Shils (1973, p. 414) discuss the controversial aspects of citral toxicity.

C. Licorice (*Glycirrhiza glabra*)

Hypertension associated with salt and water retention leading to edema follows licorice ingestion to excesses over time (Koster and David, 1968). Koster's patient ingested 100 grams per day. The noxious agent is a steroid-like compound called *glycirrhetinic acid*.

D. Cheese, Drugs, Wine and Beer

Monoamine oxidase inhibitors are in widespread use to elevate the mood. *Tyramine*, present in certain brands of cheese, wine and beer, can provoke a hypertensive crisis in patients treated with monoamine oxidase inhibitors. It takes only 6 mg of tyramine to provoke hypertension in such situations (Sen, 1969), and 25 mg (which occur in many foods or alcoholic beverages) is a very dangerous level (Hutchison, 1968).

The ripening of cheese breaks down casein, one product being tryosine. Bacteria involved convert tyrosine to tyramine. A cheese and wine party could be disastrous if high tyramine substances such as cheddar cheese and Chianti wine were freely consumed. There are other high tyramine cheeses as well as wines and beers (Sen, 1969; Bassuk and Schoonover, 1977, p. 41; Maga, 1978). Tyramine releases pressor catecholamines (Editorial, 1965).

It is interesting that some varieties of ripe cheddar and Cheshire cheese, plain chocolate, and to a small extent some wines, precipitate migraine attacks and this is due to *2-phenylethylamine* (Chaytor et al., 1975).

FOODS DISTURBING NEUROLOGIC MECHANISMS

Certain foods are cholinesterase inhibitors and thus disturb nerve impulse transmission. Such foods include potatoes (*solanine* effect), roots of tomatoes, beets, apples and eggplants (Salunkhe and Wu, 1977). Bananas in excess affect transmission of impulses because they contain serotonin and catecholamines.

Some foods produce hallucinations. Examples are nutmeg seeds and mace, which contain *myristicin*.

The methylated xanthines in tea, coffee, cocoa and cola drinks overstimulate sensitive individuals. The drugs include *caffeine*, *theophylline* and *theobromine*.

The neurologic effects of coffee are very controversial. A 5-ounce cup of coffee contains up to 90 mg of caffeine which can produce nervous excitement and cardiac stimulation, but only in a sensitive individual. Ingestion of one gram (11 cups) could seriously affect such an individual. The potential toxicity of caffeine is offset by the fact that when ingested in ordinary amounts it does not accumulate excessively in the body.

OTHER ASPECTS OF FOOD POISONING

Our discussions hardly scratch the surface. There is a large literature on food and drug interactions, making physicians take caution when prescribing antibiotics, anticoagulants, tranquilizers, and certain laboratory tests. The literature on foods as allergens is extensive. Problems in irradiation of food are still greatly unsolved. Food contamination by microorganisms is a continual and severe threat. The poisoning of animals by food toxicants and the transmission in the food chain to man is under perpetual surveillance.

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Response of Three Soybean Cultivars to Different Seed Rates

A. Goli and F. Olsen
Department of Plant and Soil Science
Southern Illinois University
Carbondale, IL 62901

ABSTRACT

Proper soybean [*Glycine max* (L.) Merr.] seeding rates are important as production costs may be increased and yields reduced by seeding at excessive rates. A field trial was conducted to determine the effect of different seeding rates of three soybean cultivars on stand density, plant growth parameters, and seed yield. The cultivars 'Amsoy 71', 'Williams', and 'Essex' (maturity groups II, III, and V, respectively) were seeded at four rates (150, 300, 450, and 600 thousand seed/ha) in 75-cm rows. The soil was a Wier silt loam classified as a fine, montmorillonitic, messic Typic Ochraqualfs. Parameters measured were stand density, plant height, lowest pod height, branch and pod numbers per plant, and yield. Soybean plant densities at maturity, expressed as a percentage of the seeding rates, were reduced each year as a result of low emergence due to severe soil crusting. Emergence was greatest for the small-seeded cv. Essex. Soybean plant height was dependent on the cultivar. The cultivar Williams grew taller then cvs. Amsoy 71 or Essex. Plant height and lowest pod height tended to increase with higher seeding rates. 'Essex' set its lowest pods higher from the soil surface compared with other cultivars. There was a consistent decrease in branch number as seeding rates increased. Pod number per plant generally increased with increasing branch number. Soybean yields were influenced by differences in stand density and pods per plant. The 'Essex', the highest-yielding cultivar in 1980, had the greatest stand density and largest pod number per plant. Planting soybeans at 150,000 seed/ha generally gave the lowest yields, but there was little advantage in sowing beyond 300,000 seed/ha.

INTRODUCTION

Soybeans [*Glycine max* (L.) Merr.] have the ability to make adjustments to different plant spacing within and between rows. For a given row spacing, however, it is important to determine the most suitable seeding rate for optimum yield. The quantity of seeds to be sown per hectare (ha) may vary depending upon cultivar, seed viability, planting method, soil type, and tillage practices used (Morse et al. 1949 and Scott and Aldrich 1970). Because so many factors are in-

volved, no standard rule has been given for the exact quantity of seeds under all conditions. Cartter and Hartwig (1962) concluded, however, that planting rates of 20 to 60 viable seed/m of row were generally satisfactory for all production areas. Buttery (1969) found that a high soybean planting density (32 plants. m^{-2}) produced larger plants with low dry weight per unit area. Soybean seed yield was reported to be less for thick than thin sowing because in the former, the lower leaves that feed the pods were shaded (Black and Watson 1960). An experiment conducted in tropical Tanzania (Enyi 1973) showed that bean yield decreased by 38, 51, and 72% when the population was increased from 74 to 111, 222, and 444 thousand plants/ha, respectively.

High plant population may occasionally result in higher yields. Lueschen and Hicks (1977) observed significant yield response from higher plant populations one year out of three in a study with three soybean cultivars. Wiggins (1939) noted that within wide ranges, the number of plants m^{-2} had little effect on net yield. Therefore, there was nothing to be gained by seeding beyond a given optimum.

Cooper (1977) and Dominguez and Hume (1978) found that soybean lodging, plant height, and lowest pod height increased with increasing plant population, whereas plant maturity, branching and pod number per plant varied in the opposite direction. The multiple effects of planting patterns on the soybean plant are cultivar dependent. According to Doss and Thurlow (1974), average yields were influenced more by cultivar or irrigation than by row width or population. Hicks and Bernard (1969) found that tall determinate types of soybeans yielded 4.6% more than short determinate types although the latter did not lodge. Osaf (1977) noted that a tall soybean cultivar produced three times as many filled pods on the main stem as a short one. This difference increased with higher populations.

Soil crusting is also an important factor which may influence soybean emergence and production. Raindrop impact on bare soils can result in severe soil crusting which may inhibit seedling emergence. Taylor (1971) observed that high soil crust strength reduced the proportion of seedlings that emerged. Yield reductions occurred when adequate plant populations were not established or where the soil crust caused plants to undergo substantial water or nutrient stress.

The influence of seeding rates on stand densities has not been fully investigated. The objective of this study was to evaluate the effects of different seeding rates of three soybean cultivars on stand density, growth, and productivity.

MATERIALS AND METHODS

This experiment was conducted at the Cooperative (Southern Illinois Univ.-Univ. of Illinois) Agronomy Research Center at Carbondale, IL. in 1979 and 1980. Three soybean cultivars 'Amsoy 71', 'Williams' and 'Essex' were evaluated. For the latitude of this study, these cultivars are early, medium, and full season maturity (group II, III, V), respectively. Essex is a determinate cultivar and the other two are indeterminate. Four seeding rates (150, 300, 450, and 600 thousand seed/ha) were used for each cultivar with 75-cm row spacing. Planting was done with an Allis Chalmers No-Till planter on 25 May 1979 and 15 May in 1980, respectively. Seed germination tests (warm and cold) were made before planting (Table 1). A new supply of soybean seed was obtained each year for each of the soybean cultivars. The soil was a Weir silt loam classified as a fine, montmorillonitic, mesic Typic Ochraqualfs. The soil had an organic matter content of 1.5%. Initial

soil pH was 6.7 and soil test available P was high and exchangeable K very high. No lime was applied during the study. A topdressing of 15 kg P/ha (0-46-0) and 84 kg K/ha (0-0-60) was applied in 1979. Weed control was accomplished in 1979 with 1.1 kg/ha of Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) and 1.8 liters/ha of Trifluralin (α,α,α, Trifluro-2,6-dinitro-N,N-diprophyl-p-toluidine). In 1980, 4.6 liters/ha of Alachlor (2-chloro-2, 6′-diethyl-N-(methoxymethyl) acetanilide) and 1.6 kg/ha of Linuron were applied.

A randomized complete block experimental design with three replications was employed. Individual plots were 3 m by 12 m. Two inner rows within each four-row plot were used for data collection. Data was analyzed using the analysis of variance, and significant means were separated with Duncan's Multiple Range Test.

The following parameters were evaluated: 1) stand density, 2) plant height from ground level to the tip of main stem at maturity, 3) height from soil surface to the lower tip of the lowest pod, 4) average number of branches and pods per plant, and 5) seed yields adjusted to 13% moisture.

RESULTS AND DISCUSSION

Stand counts. Soybean stand densities were influenced by cultivars and seeding rates. Greater seeding rates generally resulted in higher stand densities at harvest. Final stand densities at maturity (expressed as a percentage of the seeding rate) indicated that seedling emergence and survival were low (Fig. 1 and 2). This can be attributed to soil crusting caused by rains following planting in a conventional seedbed in both years of the study. The beating rains on the low organic matter soils commonly found in souther Illinois, southern Indiana, and western Kentucky break down the soil structure resulting in severe crusting. Penetrometer readings were taken to evaluate soil crust strength after rains. The pressure required to break the soil crust for three successive days following a rain was 2.04, 3.42, and 4.15 kg/cm^2, respectively. This progressive increase in soil crust strength made seedling emergence difficult.

There were no notable differences in stand density among soybean cultivars in 1979 (Fig. 1). However, in 1980, the cv. Essex had a higher stand density than the cvs. Amsoy 71 or Williams (Fig. 2). Other factors besides genetic differences among soybean cultivars affect seedling emergence. Two such factors are seed viability and seed size (Morse et al. 1949). The cold germination test indicated that the cv. Essex seed should germinate and emerge better under stress conditions than the cvs. Amsoy 71 or Williams (Table 1). This may account for some of the differences in seedling emergence observed among cultivars. Additionally, the greater seedling emergence of the cv. Essex may be related to smaller seed size. Small seed size may be advantageous in cases of soil moisture stress and soil crusting. These two factors were especially severe in 1980.

Variation in seeding rates of each cultivar did not affect the percentage of mature plants during 1979 (Fig. 1). However, in 1980, the seeding rates of the cvs. Williams and Essex did influence the final plant population. The percentage of mature plants tended to increase with higher seeding rates. The percentage plant population of the cv. Essex increased with seeding rates up to 450,000 seed/ha. When the seeding rate of this cultivar was low, the seedlings were further apart within the row, and thus, were unable to provide mutual assistance in breaking

the soil crust. At 600,000 seeds/ha, more seedlings emerged but subsequent competition for space within the row may have reduced seedling numbers.

Plant height. Plant height variations differed significantly among soybean cultivars (Table 2). The cv. Williams tended to have the tallest plants, whereas the cvs. Essex and Amsoy 71 had lower but similar plant height. Soybean plant vegetative growth varies with different latitudes because of its dependence on temperature and photoperiod. A given soybean cultivar usually grows taller as latitude increases. The cv. Amsoy 71 is a maturity group II cultivar which is better adapted to latitudes north of the region where this study was conducted. Shorter plants probably resulted because Amsoy 71 was grown in a more southern latitude in this study. The experimental location was well suited to the cv. Williams, a maturity group III cultivar. The cv. Essex was expected to grow taller than it would in its best zone of adaptation. Soybean plant heights decreased by 27% from 1979 to 1980 when averaged over cultivars and seeding rates. Lower rainfall and higher temperatures during the summer of 1980 (Table 4) may have contributed to the shorter soybean plants. Plants tended to be taller as seeding rates increased (Table 3). This may have occurred because soybean plants compete for light more at higher densities.

Height of the lowest pod. In 1979, the cv. Essex set its lowest pod much higher from the soil surface than the other two cultivars (Table 2). Again in 1980, the cv. Essex set its lowest pod much higher than the other cultivars. Lowest pod height generally increased with increasing seeding rates (Table 3). Although the trend was consistent in both years, the lowest pods tended to be nearer to the soil surface in 1980 than in 1979 when compared at the same seeding rate. It is possible that the cv. Essex is genetically predisposed to set its lowest pod at a higher level from the soil surface than the cvs. Amsoy 71 or Williams. Other determinate soybeans like the cv. Essex need to be evaluated before this difference can be attributed to the determinate character.

Plant branching. The early-maturing cv. Amsoy 71 generally had more branches per plant than the late-maturing cv. Essex (Table 2). Plant branching of the cv. Williams was similar to the cv. Essex in 1979. All three cultivars had more branches and were shorter in 1980 than in 1979. Branch numbers increased as stand densities decreased (Table 3). At lower stand densities, less competition among plants may have resulted in the production of more branches. Lower stand densities resulted in a great number of branches per plant for all cultivars in 1980 compared with 1979.

Pods per plant. The cv. Essex produced the greatest number of pods per plant in both years (Table 2). No statistical differences were observed in pod numbers per plant between the cvs. Amsoy 71 and Williams. The number of pods per plant tended to decrease with higher seeding rates, with the greatest decrease occurring from 150,000 to 300,000 seed/ha (Table 3). There was no notable difference in pod numbers per plant when sowing 450,000 or 600,000 seed/ha.

Soybean yields. The cv. Essex outyielded the cv. Williams in 1980 but not in 1979 (Table 2). 'Amsoy 71' was the lowest yielding cultivar both years. Soybean yields tended to increase with increasing days to maturity, especially with unfavorable climatic conditions. The period from planting to maturity of the cvs. Amsoy 71, Williams, and Essex was 108, 128, and 149 days, respectively, in 1980. Yields were less in 1980 than in 1979 by 59, 51, and 38% for cvs. Amsoy 71, Williams, and Essex, respectively. The relatively low yields obtained in 1980 were attributed primarily to high temperature and dry conditions during the major period of

vegetative growth and seed filling (Table 4). Additionally, rainfall distribution was concentrated in the early period of plant growth (Table 4). During both years, planting 150,000 seeds/ha gave the lowest yields (Table 3). There was no significant yield advantage in seeding beyond 300,000 seeds/ha in either year. The lowest yield each year was from the cv. Amsoy 71 planted at 150,000 seed/ha.

Correlations among parameters measured. In general, soybean yields were positively correlated with plant heights, lowest pod heights, stand densities and seeding rates (Table 5). The positive correlation of yields with lowest pod heights may be accounted for by reduced seed loss during harvest because pods were set higher. Soybean yields were negatively correlated with branch number per plant probably because branch and pod numbers per plant were negatively correlated with stand density. Greater branch or pod numbers per plant were accompanied by lower stand densities and lower yields. Soybean plant height was found to be negatively correlated with branch number and pod number per plant. Soybean plant heights were positively correlated with stand densities, and seeding rates. Higher stand densities from increased seeding rates resulted in severe competition among plants and greater plant heights. Branches and pods per plant were highly positively correlated. The numbers of branches per soybean plant were negatively correlated with stand densities and seeding rates. The number of pods per plant tended to be negatively correlated with stand densities and seeding rates and the lowest pod heights were positively correlated with stand densities and seeding rates.

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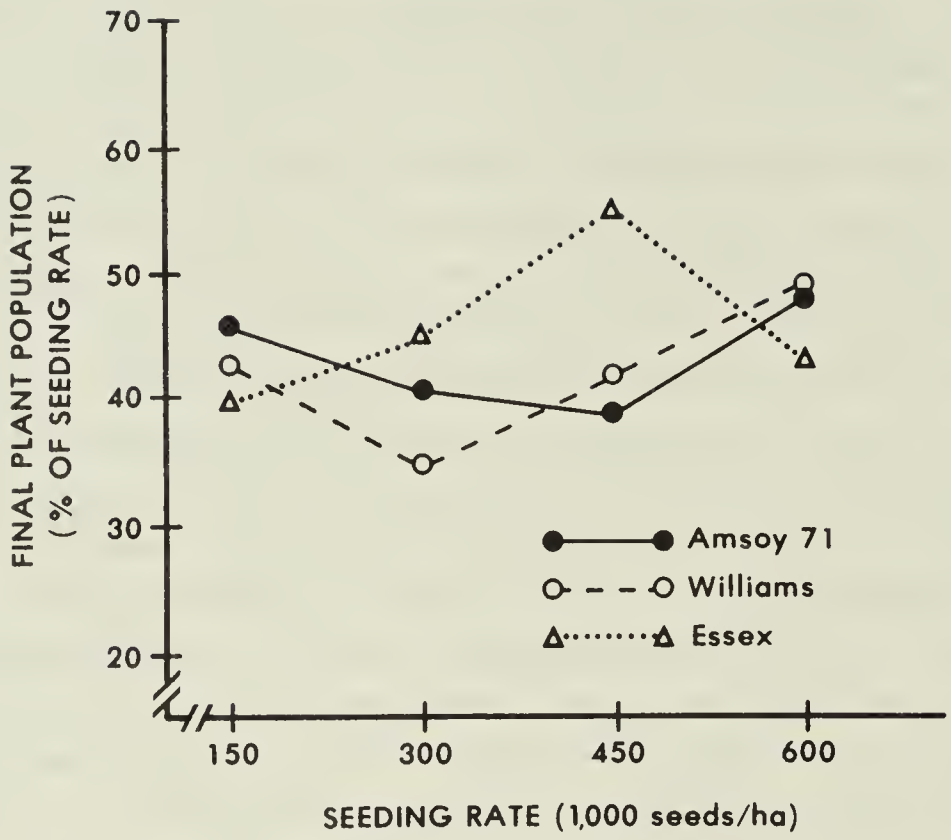

Fig. 1. Relationships between seeding rate and the percentage of final plant population of three soybean cultivars during 1979.

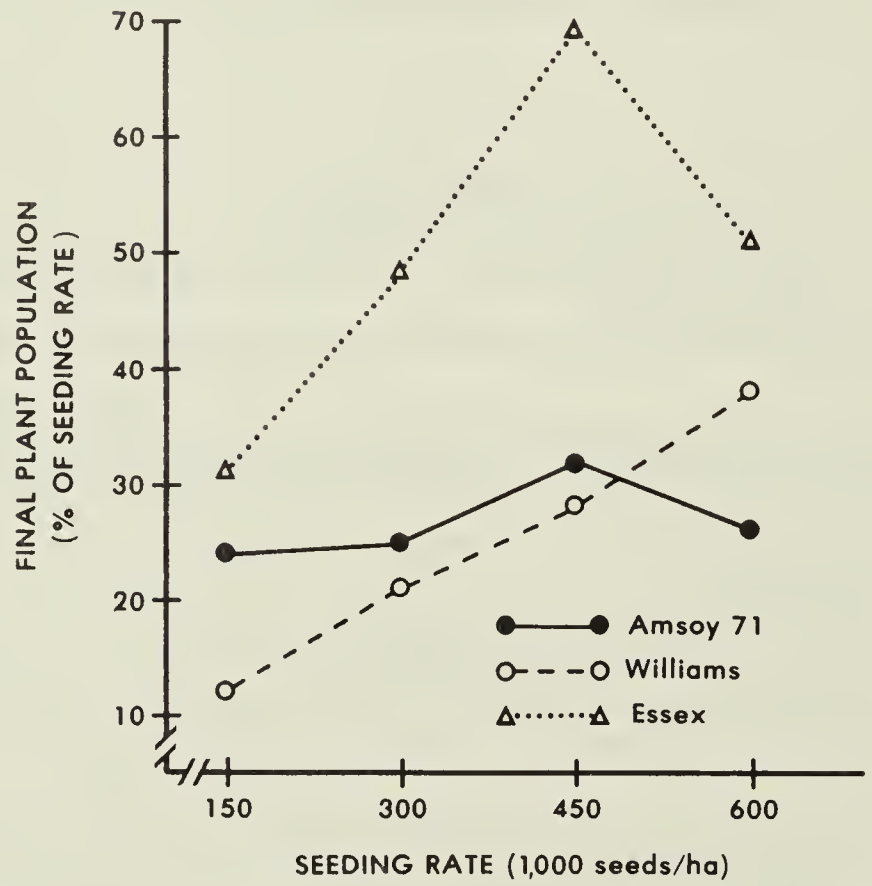

Fig. 2. Relationship between seeding rate and the percentage of final plant population of three soybean cultivars during 1980.

Table 1. Germination tests and seed size prior to planting three soybean cultivars in 1979 and 1980.

Cultivars	Warm tests 1979	Warm tests 1980	Cold tests 1979	Cold tests 1980	Seed sizes 1979	Seed sizes 1980
	---------- % germination ----------				---g/100 seeds---	
Amsoy 71	100	97	46	48	19.6	19.6
Williams	100	98	38	68	17.6	18.1
Essex	99	99	63	79	15.7	14.0

Table 2. Effects of soybean cultivars on plant heights, lowest pod heights, branches per plant, pods per plant and yields during 1979 and 1980.

	1979					1980				
Cultivars	Plant Heights	Lowest Pod Heights	Branches	Pods	Yields	Plant Heights	Lowest Pod Heights	Branches	Pods	Yields
	----------cm----------		----number/plant----		---kg/ha---	----------cm----------		----number/plant----		---kg/ha---
Amsoy 71	74b*	3.5b	3.9a	71b	1,982b	52b	2.4b	4.5ab	68b	790c
Williams	86a	3.7b	3.0b	63b	2,466a	63a	3.2b	4.8a	59b	1,054b
Essex	77b	13.2a	3.3b	99a	2,486a	55b	8.5a	3.7b	92a	1,522a

*Means in the same column with the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

Table 3. Effects of soybean seeding rates on plant heights, lowest pod heights, branches per plant, pods per plant and yields during 1979 and 1980.

	1979					1980				
Seeding Rates	Plant Heights	Lowest Pod Heights	Branches	Pods	Yields	Plant Heights	Lowest Pod Heights	Branches	Pods	Yields
seeds/ha × 10^3	----------cm----------		----number/plant----		---kg/ha---	----------cm----------		----number/plant----		---kg/ha---
150	71b*	4.1b	5.6a	119a	1,966b	54a	2.1c	8.0a	138a	858b
300	79ab	5.5ab	3.7b	78b	2,297a	55a	4.4b	4.4b	57b	1,133a
450	81ab	6.9a	2.4c	62c	2,369a	59a	5.3ab	3.9b	51bc	1,218a
600	83a	6.5a	2.2c	53c	2,456a	59a	5.7a	2.9c	42c	1,162a

*Means in the same column with the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

Table 4. Monthly mean maximum and minimum temperatures and rainfall from May-Sept. 1979 and 1980 at Carbondale, Il.

	1979			1980		
	Temperature			*Temperature*		
Month	Max	Min	Rainfall	Max	Min	Rainfall
	-------C°-------		cm	-------C°-------		cm
May	24.9	10.1	8.0	25.7	11.6	7.3
June	30.1	15.1	9.3	31.4	15.7	15.0
July	30.8	19.2	14.3	35.1	21.1	11.0
Aug.	30.6	26.1	10.9	34.9	20.9	3.0
Sept.	27.5	11.6	3.7	30.5	16.6	8.7

Table 5. Correlation coefficients among soybean parameters during 1979 and 1980.†

	Yields	Plant Heights	Branches	Pods	Lowest Pod Heights	Stand Densities
Plant Heights	0.63**					
	0.28					
Branches	-0.55**	-0.54**				
	-0.63**	-0.20**				
Pods	-0.23	-0.56**	0.73**			
	-0.30	-0.49**	0.76**			
Lowest Pod Heights	0.38*	-0.02	-0.30	0.16		
	0.84**	0.15	-0.62**	-0.27		
Stand Densities	0.34	0.38*	-0.84**	-0.68**	0.31	
	0.59**	0.35*	-0.75**	-0.68**	0.81**	
Seeding Rates	0.93*	0.93*	-0.95*	0.95*	0.89*	-0.27
	0.80	0.96**	-0.92*	-0.85	0.94*	0.77

*Significant at the 5% level.
**Significant at the 1% level.
†Top numbers are for 1979 and bottom numbers for 1980.

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Midwest Plants for Potential Crops[1]

Irene M. Cull, Botanist
Northern Regional Research Center,
Agricultural Research Service,
U.S. Department of Agriculture,[2]
Peoria, Illinois 61604

ABSTRACT

In the fall of 1975, the Northern Regional Research Center expanded its plant screening program to include midwestern plants for sources of rubber and other hydrocarbons, fiber, oil, and protein. More than 600 species have been collected. Of these, 515 species, representing 288 genera and 86 families, have already been analyzed and the results on 200 species have been published. Results on 200 more have been reported at scientific meetings (1980 and 1981). For each species a cumulative score was calculated, partly from the botanical characteristics and potential adaptability to cropping and partly from the chemical composition. Seventy-three species ranked 11 or less; therefore, they may be amenable to cropping and product utilization. These species should be studied further.

INTRODUCTION

Plants as sources of rubber have been investigated intermittently for many years in North America and Europe. The main reason for these efforts is the continuous need for importing natural rubber from South East Asia and South America. Annual U.S. imports alone are as high as 1.6 billion pounds. So far, all efforts to match the excellent properties of the natural product with petroleum-based or other manmade rubbers have failed. The first documented experiments to prepare rubber from U.S. plants were begun in 1871 by William Saunders. He used Common Milkweed (*Asclepias syriaca* L.) and published his report in 1875. Cook (1903) worked on the Central American Rubber Tree (*Castilla elastica* Sesse) and on Guayule (*Parthenium argentatum* A. Gray). A.T. Saunders (1910) examined 1,900 milkweed plants as sources of rubber. In his report he includes characteristics of the rubber. The most extensive agronomic examination of milkweed plants up to 1912 was done by Fox (1911 and 1912). Great Basin and Pacific Coast plants were analyzed in California in 1917. Results were published by Hall and Goodspeed

[1]Presented at Illinois State Academy of Science Meeting, Lisle, IL, April 18-19, 1980.

[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

(1919). Most of their work was on varieties of Rabbit-brush [*Chrysothamnus nauseosus* (Pall.) Britt.]. Pearson (1919) learned that the Germans during World War I resorted to the manufacture of rubber from weeds, e.g., Sow Thistle (*Sonchus* spp.) and Wild Lettuce (*Lactuca* spp.).

In 1917, a project on the rubber content of North American plants was begun as a war emergency project. Most of this work was done on Rabbit-brush [*Chrysothamnus nauseosus* (Pall.) Britt.] (Hall and Long, 1921), but 64 species of milkweed were examined, too. Their report also includes a list of composites that gave negative results. Widstoe and Hirst (unpublished manuscript, mentioned by Hall and Long, 1921) of Utah determined the percentage of rubber in several species and prepared rubber for examination. Also according to Hall and Long (1921), Olsson-Seffer, an experienced student of rubber plants, studied Mexico latex plants, especially *Jatropha*, *Plumeria*, and *Euphorbia* species. He learned that these three genera have the ability to reproduce from the base after the plant tops are removed. He also realized that commercial quantities of rubber could be obtained from native growth and from plantations established for this purpose. However, before his work could be published in detail, he was killed in a political disturbance in Mexico.

Thomas Edison searched for rubber-producing plants from 1927-1931 and tested products of thousands of plants. Polhamus (1967) reported the results. Edison analyzed 2,222 species representing 977 genera and 186 families. Much of his work was on goldenrods (*Solidago* spp.).

Again during World War II there was an increased effort to search for rubber-producing plants. A report published in 1945 includes a table giving the rubber content of native plants of Arizona (Anonymous, 1945). All these efforts were discontinued either because they did not appear to result in economically feasible products or because the emergency situations ended. However, during the last decade the need for additional efforts has resurfaced, since world production of natural rubber is expected to be insufficient for the demand. Also, strategic considerations play a major role, since present production sites are so distant from the major users of natural rubber.

Until now, midwestern species of plants have never been included specifically in the search for rubber and other hydrocarbons. Since 1975 the Northern Regional Research Center has been screening such midwestern plants as sources of hydrocarbons, with special emphasis on rubber, oil, and wax. Reports have been published on 200 species (Buchanan *et al.*, 1978 [4 papers]). Reports on an additional 200 species have been presented at scientific meetings in 1980 and 1981, respectively. These reports will be published soon. Approximately 100 species analyzed were legumes from many parts of the world. Analyses on the last 100 species are completed but they are not yet evaluated and organized for presentation.

At first the search was directed toward three groups of plants: (1) those with milky juice producing natural rubber, (2) local plants of the same families and genera included in the survey done at the Arizona Experiment Station in the 1940's, and (3) species analyzed by Edison in his search for rubber-producing plants. Because of the richness of the Midwestern flora, there are hundreds of plant species that can be obtained in most local areas. For example, a short-term survey of a limited area along an abandoned railroad right-of-way yielded more than 500 plant species (Cull, 1969).

MATERIALS AND METHODS

Collections

More than 600 species of mature plants from the midwest have been collected for chemical analyses up to December 1, 1982. A voucher specimen of each species has been preserved in our Center's herbarium. A 500-gram sample of each species, usually composed of several plants or twigs, was collected at one location. If the plants were small, they were collected at several places until the desired amount was obtained. Herbaceous and small woody plants were clipped at the soil line. New growth was clipped from large woody plants. Where possible or desirable, material included flowers and/or fruits and seeds. Individual species were collected in a large paper sack. On the sack and in a field notebook were written the common or scientific name and the date and place of collection. Pertinent information about each sample was provided to the analytical chemist. This included scientific name, common name if there was one, family, collector, place and date of collection, and sometimes additional notes where deemed desirable.

Gray's Manual was the source for the scientific names and authorities (Fernald, 1970).

Drying

The green material was air-dried indoors, usually in the sacks. However, large plants and woody ones were spread on plastic sheets on the floor or a table top to dry. The degree of dryness was judged satisfactory if the leaves crumbled when squeezed and the stems snapped when bent.

Sample Grinding and Analysis

When dry, each sample was coarsely chopped and ground to 1 mm sieve size and analyzed by chemists. The analyses included oil, polyphenols, hydrocarbons, and protein. Also, the presence of natural rubber, wax and fiber was noted.

Botanical Evaluation

Plants were evaluated on the basis of their botanical characteristics. Each species was scored in each of five categories, namely, temperature requirement (scoring range 1-5), moisture requirement (1-5), growth rate (1-4), plant habit (1-5), and adaptability to pollarding (1-3). Lowest numbers indicate the best possibilities for crop development. Table 1, developed for country-wide use, served as a guide. Plants that score high but have hydrocarbon-rich fruits, roots, rhizomes, bulbs, etc., or which are lactiferous and can produce hydrocarbons by tapping, are scored one number lower on the scale than their botanical characteristics indicate. Growth rate for perennials, including woody species, has been defined as the amount of annual growth after clipping at ground level. A score of 4 applies to all species of vines, marsh plants, rosette-forming plants, low-growing succulents and sod-forming grasses, including those with a high rate of growth. An ideal candidate would score 5 and a useless one 22. The total botanical rank for each species was used in the cumulative rating by dividing the score by 5, rounding to the nearest whole number, and adding the result to the total chemical evaluation score. Any species having more than one undesirable characteristic was rated 5 on this reduced botanical scale.

Chemical Evaluation

Plants also were evaluated on the basis of their chemical composition. Each species was ranked in each of four categories, namely, fiber (score of 1 or 2), pro-

tein (1 or 2), oil (1 through 4), and hydrocarbons (1 through 4) (Table 2). Plants potentially useful for making fiber, paper, or board products were rated 1, all others were rated 2. Species that contained more than 14% protein were rated 1, all others were rated 2. Oil fraction over 8% was rated 1; 5-8%, 2; over 2-5%, 3; and less than 2%, 4. Hydrocarbon content was rated likewise. Species with more than 2% hydrocarbons were rated 1; 1.2 to 2.0%, 2; 0.4 to 1.2%, 3; and less than 0.4%, 4. Each species was then ranked in each of four categories, namely, fiber, protein, oil, and hydrocarbons. This approach was chosen to give additional consideration to those plant species whose total biomass may be utilized for the manufacture of desirable products.

The total scores were used in determining the cumulative rating.

Cumulative Rating

A cumulative rating was calculated for each species, based on the sum of the reduced botanical rating and the chemical evaluation (crude protein, fiber, percentage of oil and of hydrocarbon). The smaller the number, the greater the possibility for a future crop. If a species were suitable for oil or hydrocarbon but not both, it would score 9. Species having a cumulative score of 11 or less appear to have potential and warrant further study. It should be understood that this cumulative rating system is not perfect, but that it can be used satisfactorily to identify the more promising species.

RESULTS AND DISCUSSION

The first 515 species analyzed represented 288 genera and 86 families. All species analyzed contained hydrocarbons, but the amount was often very small. Plant families containing the most hydrocarbon-producing midwestern species analyzed are shown in Table 3. Natural rubber is found in 92 Midwestern species. The highest concentration levels of natural rubber occurred in the family *Asclepiadaceae*, the milkweeds. The species analyzed were Common Milkweed (*Asclepias syriaca* L.), Swamp Milkweed (*A. incarnata* L.), Whorled Milkweed (*A. verticillata* L.) and Butterfly Weed (*A. tuberosa* L.). Common milkweed (*A. syriaca* L.), the largest of the milkweeds, contained the most rubber.

Twenty-one species of the midwestern plants analyzed contained more than 1% hydrocarbon (Table 4).

Pale Indian Plantain (*Cacalia atriplicifolia* L.) had the highest percentage of hydrocarbon. Also the seed oil contains dammarenediol II esters (Spencer 1981). This species grows well in sand and in prairie soil and is widely distributed east of the Mississippi River.

Wild Bergamot (*Monarda fistulosa* L.) was an outstanding rubber-producing plant, having a large amount of high-quality rubber. Abandoned fields colonized by Wild Bergamot indicate it could be grown like hay.

Natural rubber is found also in Compass Plant (*Silphium laciniatum* L.), Giant Ragweed (*Ambrosia trifida* L.), Sow Thistle (*Sonchus arvensis* L.), Common Elder (*Sambucus canadensis* L.), Indian Hemp (*Apocynum cannabinum* L.), New England Aster (*Aster novae-angliae* L.) and Stiff Goldenrod (*Solidago rigida* L.).

Tall Bellflower (*Campanula americana* L.) in addition to high natural rubber content is high in oil and protein.

Pokeweed (*Phytolacca americana* L.) contains more than 15% protein.

Sassafras [(*Sassafras albidum* (Nutt.) Nees] is high in oil and grows rapidly even on poor soil.

Our survey indicates that many midwestern plants have potential as future crops. Hydrocarbon crops that also produce fiber, oil, and protein can be developed. The analyses indicate that a common weed, toothed spurge (*Euphorbia dentata* Michx.), as it is today, could produce more oil and protein per specified area than soybeans or alfalfa. This spurge can produce 649 kg/ha/yr of oil and 1,223 kg/ha/yr of protein, whereas soybeans yield 347 kg/ha/yr of oil and 743 kg/ha/yr of protein, and alfalfa 148 kg/ha/yr oil and 1,096 kg/ha/yr, respectively (Buchanan, 1978). Although it may have toxic properties, since many plants of the Euphorbiaceae do, it could still serve as an energy crop. Perhaps the protein could be isolated from the toxins, or the toxins might be removed from the plants through breeding.

Factors to Consider in Developing New Crops

Many factors must be considered when developing a new crop. Some of these are shown in Table 5. Large plants would be better than small ones, because more material could be produced on an equivalent amount of ground. Upright plants are easier to harvest than mat types or vines. A mesic habitat is easier to work than either wet or very dry habitats. Plants that thrive in various types of soil lend themselves to wider geographical distribution. The higher the concentration of the end-product in the plant or its components, the more useful it should be. Sometimes fertilizers or other chemical treatment can increase a desired component in a crop plant. Yokahama has found that chemical stimulants can increase rubber content in plants two- to sixfold (Anonymous, 1977).

Seed germination may require certain special conditions, such as a cold period for a certain length of time before germination. In some species, such as sunflower and milkweed, components naturally present in the seed or seed coat may hinder germination or kill the seedlings. Allelopathic properties may have to be considered for some species. Often there is more genetic variability in a wild species than in a cultivated one.

Methods of processing the material and utilization of the by-products are necessary. We will have to learn whether these plants have diseases and insect pests when grown as crops. Here in Illinois, the Common Milkweed (*Asclepias syriaca* L.) appears to be a healthy species without serious insect pests, even though many kinds of insects visit it. However, there may be serious problems when the species is grown in monoculture or in a different part of the country. Dr. Yokahama from California obtained seeds and plants of Common Milkweed (*A. syriaca* L.) from us in early spring. He grew them and found that California insects liked this species so much that he was unable to raise enough material for his research.

Another problem in developing a new crop from local plants will be convincing farmers that they should grow species now considered weeds on which much time and many dollars have been spent for eradication. Whether these plants will grow as well in cultivation as they do in the wild remains to be seen.

FUTURE RESEARCH

Much research will have to be done before some of our wild plants can be grown as profitable crops (see Table 6). Agronomic research on all phases of crop

production is required (planting, cultivating, fertilizing, harvesting). Diseases and insect pests must be determined and methods of controlling these must be found.

Genetic research will be necessary: (1) to produce plants of uniform size that will mature at the same time, (2) to increase the concentration of the desired products, (3) to develop resistance to diseases and insect pests.

Other areas of research required include transportation, storage, processing, and by-product and end-product utilization.

SUMMARY

Since 1975, more than 600 species of plants have been collected to search for sources of hydrocarbons, especially natural rubber and wax. Of these species, 515 representing 86 families and 288 genera have been analyzed. All species contained hydrocarbons. Many of those species with high hydrocarbon content, especially natural rubber, have characteristics that make them suitable for future crops. Some of the more promising common weeds appear, indeed, to be good prospects for new crops. However, many factors must be considered in developing these species as crops, and a vast amount of research needs to be done in many areas before these plants can become profitable crops.

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Table 1. Botanical Rating

Parameter	Rating 1	2	3	4	5
Temperature requirement	Zone 1-2-3	4-5	6-7	8-10	Tropical
Moisture requirement	Mesic	Mesic	Subxeric	Xeric, marsh	Aquatic
Growth rate, m	> 1.5	1.0-1.5	0.5-1.0	< 0.5	---
Plant habit	Tall upright	Short upright	Mat, prostrate	Vines,[a] etc.	Epiphytes and parasites
Adaptability to pollarding	Multiple annually	Once annually	No	---	---

[a]Includes vines, marsh plants, rosette-forming plants, low-growing succulants, and sod-forming grasses.

Table 2. Rating of Plant Species According to Their Composition

Component	Rank 1	2	3	4
Fiber	Suitable for fiber, paper or board	Not suitable for making fiber, paper or board products	---	---
Crude protein	> 14%	< 14%	---	---
Oil fraction	> 8%	5-8%	2-5%	< 2%
Hydrocarbon fraction	> 2%	1.2-2.0%	0.4-1.2%	< 0.4%

Table 3. Midwest Plant Families With Most Hydrocarbon-Producing Species (First 300 midwest species analyzed)

Family	Species analyzed	Number of species with natural rubber and/or wax	Percent of species showing promising results
Asclepiadaceae	11	10	90.9
Caprifoliaceae	8	7	87.5
Apocynaceae	4	3	75.0
Labiatae	34	21	61.7
Euphorbiaceae	17	10	58.8
Compositae	85	49	57.6
Gramineae	29	9	31.0

Table 4. Midwest Species Containing More Than 1% Hydrocarbons

Scientific name	Common name	Percentage
Cacalia atriplicifolia L.	Pale Indian Plantain	3.10
Asclepias tuberosa L.	Butterfly Weed	2.54
Agropyron repens (L.) Beauv	Quack Grass	1.72
Lonicera tatarica L.	Tartarian Honeysuckle	1.68
Asclepias syriaca L.	Common Milkweed	1.59
Humulus japonicus Sieb. and Zucc.	Japanese Hop	1.53
Teucrium canadense L.	American Germander	1.44
Solidago graminifolia (L.) Salisb.	Grass-leaved Goldenrod	1.43
Artemisia vulgaris L.	Common Mugwort	1.41
Solidago rigida L.	Stiff Goldenrod	1.39
Triosteum perfoliatum L.	Tinker's Weed	1.33
Aster laevis L.	Smooth Aster	1.31
Elymus canadensis L.	Wild Rye	1.28
Pycnanthemum incanum (L.) Michx.	Hoary Mountain Mint	1.24
Lippia lanceolata Michx.	Fog-fruit	1.24
Phalaris canariensis L.	Canary Grass	1.22
Rudbeckia subtomentosa Pursh.	Sweet Coneflower	1.22
Monarda fistulosa L.	Wild Bergamot	1.15
Monarda punctata L.	Horsemint	1.15
Elaeagnus multiflora Thunb.	Cherry Elaeagnus	1.13
Apocynum cannabinum L.	Indian Hemp	1.01

Table 5. Factors to Consider When Developing a New Crop

Plant size	Effect of chemical stimulation on desired product
Plant habit	Germination of seed
Type of habitat	Genetic variability
Type of soil	Processing
Annual, biennial, or perennial	By-product utilization
Density of stand	Diseases
Effect of fertilizers	Insect pests
Concentration of end product in plant or its components	Convincing farmers to grow the crop

Table 6. Research Needed

Agronomy
All phases of crop production

Genetics	*Other*
Evaluation of genetic variability	Transportation
Concentration of desired products	Storage
Uniform maturity	Processing
Plant habit	End product utilization
Disease resistance	By-product utilization
Insect resistance	Chemistry

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Ultrastructural Changes in Mesophyll Cells of Developing Seed Cotyledons of Pharbitis Nil

Walter E. Riedell
Department of Botany,
Southern Illinois University,
Carbondale, Illinois 62901

ABSTRACT

The cellular ultrastructure of mesophyll cells of cotyledon tissue from seeds of *Pharbitis nil* (Japanese morning glory) was studied. As the cotyledons of the seed develop, the mesophyll cells undergo marked changes. Mesophyll cells of the seed cotyledon 17 days after anthesis appear highly vacuolated. The appearance of lipid bodies, protein bodies, and large amounts of carbohydrates within the plastids characterize the cells of the seed cotyledons 30 days after anthesis. The most striking difference between 30-day-old and 40-day-old seed cotyledon mesophyll cells is the appearance of phytin within the protein bodies of the 40-day-old seed cotyledon mesophyll cells.

INTRODUCTION

Pharbitis nil is a short-day plant that has been used to study the photoperiodic requirements which initiate flowering (Fredericq, 1963; King and Vince-Prue, 1978).

Seeds of *Pharbitis nil* have been used to study changes in gibberellin-like substances during seed development (Ogawa, 1963), and to study the effect of compounds which inhibit GA biosynthesis (Zeevaart, 1966). Although Hayward (1932) briefly mentioned the development of seed cotyledons in *Ipomoea batatas*, and Wada et al. (1981) studied the development of giant oil cells in seed cotyledons of *Pharbitis nil*, the literature contains no reports of the ultrastructure of these organs.

A developmental study of the ultrastructure of the developing seed cotyledons of this species, therefore, offered the possibility of giving additional insight into ultrastructural changes which take place during seed development. Because of this, the present investigation was undertaken.

MATERIALS AND METHODS

Seeds of *Pharbitis nil* Chois., strain Scarlett O'Hara (Burpee Seed Company, Clinton, Iowa 52732) were manually scarified and germinated within layers of moist paper towels. Upon germination, the seeds were transplanted to fertile soil in 6 inch clay pots and placed under 18-hour photoperiod in the greenhouse. After several weeks, the plants were transferred to a 12-hour inductive photoperiod. Individual flowers were marked on the date of anthesis. Cotyledons dissected from seeds 17, 30, and 40 days after anthesis were fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer at pH 7.4 for 3 hours at room temperature. The tissues were then washed with 0.15 M phosphate buffer and post-fixed in 1% osmium tetroxide in 0.15 M phosphate buffer, pH 7.4, for 2 hours at room temperature. The tissues were dehydrated in an ethanol-acetone series and embedded in Spurr (1969) low viscosity resin. Thin sections were picked up on uncoated 300 mesh copper grids and post-strained for 3 minute in Reynolds (1963) lead citrate and examined with a Hitachi HS-9 transmission electron microscope at 75 kV.

For the cytological localization of carbohydrate and protein, cotyledons dissected from seeds 30 days after anthesis were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer, pH 6.8, for 6 hours at 4 C. The tissues were dehydrated in an ethanol, n-propanol, and n-butanol series at 4 C and embedded in glycol methacrylate (Feder and O'Brien, 1968). Thick sections were stained with analine blue-black for total protein (Fisher, 1968), and with periodic acid-Shiffs reagent for carbohydrate (Feder and O'Brien, 1968), and viewed with a Leitz Ortholux light microscope.

RESULTS

Mesophyll cells of seed cotyledons examined 17 days after anthesis contain many vacuoles (Fig. 1). The presence of a large nucleus, plastids, mitochondria, dictyosomes, and cisternae of endoplasmic reticulum characterize the cellular ultrastructure of this cotyledonary tissue. The plastids display a moderately developed grana-fretwork system, some starch deposition and still show a prolamellar body (Fig. 2).

Twelve additional days of seed maturation result in dramatic changes in the seed cotyledons. Cytochemical stains specific for carbohydrate (Fig. 3) and for total protein (Fig. 4) reveal that both of these substances are present in copious amounts within the mesophyll cells. Electron micrographs of mesophyll cells the same age as those pictured in Fig. 3 - 4 indicate that areas staining positive for carbohydrate are plastids, and areas staining positive for protein are protein bodies (Fig. 5). Numerous lipid bodies occupy the peripheral region of the mesophyll cells. The protein bodies have an irregular outline and are filled with a homogenous granular/fibrillar material. All of the mesophyll cells at this cotyledonary stage of development contain protein bodies. They usually contain one or more inclusions called globoids (Fig. 5). Other cytoplasmic organelles present are mitochondria and plastids with voluminous starch grains.

Mesophyll cells of seed cotyledons that have matured for 40 days are slightly different from those of 30-day-old seed cotyledons. Globoids usually contain large electron-opaque crystals of phytin (Fig. 6). Lipid bodies, identified by their electron-transparent nature, occupy a large area of the cytoplasm. Plastids are also present in cells of 40-day-old seed cotyledons. These usually contain voluminous starch grains (Fig. 6).

DISCUSSION

Examination of photomicrographs of mesophyll cells of seed cotyledons indicates that these cells undergo marked changes during seed development. The most noticeable change is the appearance of lipid bodies and protein bodies in the mesophyll cells 30 days after anthesis. By the time the seed cotyledons reach an age of 40 days, virtually all of the cell volume not occupied by nuclei and plastids is filled with lipid bodies and protein bodies.

Protein bodies, once formed, usually contain one or more inclusions called globoids (Bewley and Black, 1978). Globoids are the site where calcuim, magnesium, or potassium salts of phytic acid (phytin) crystallize (Lott, 1975). Phytin crystals, not seen in globoids of protein bodies of 30-day-old cotyledon mesophyll cells, are abundant in globoids of protein bodies of 40-day-old cotyledon mesophyll cells.

Plastids of seed mesophyll cells contain starch grains (carbohydrate). These starch grains appear to be much smaller in the plastids of 17-day-old mesophyll cells than in the plastids of 30-day-old mesophyll cells. This indicates that starch gradually accumulates with time following anthesis.

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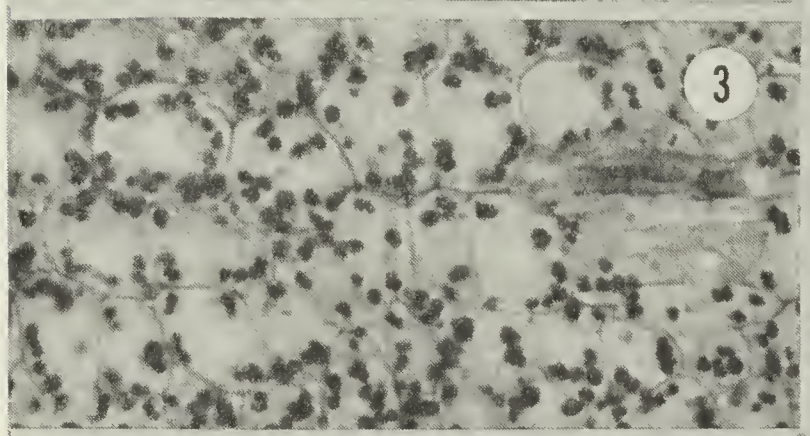
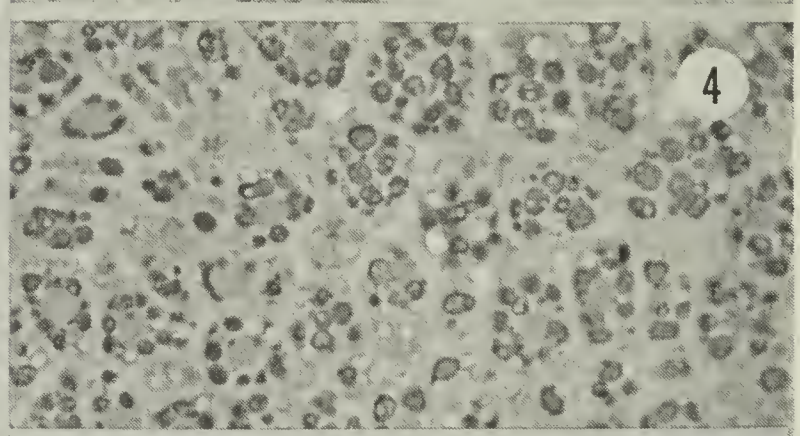
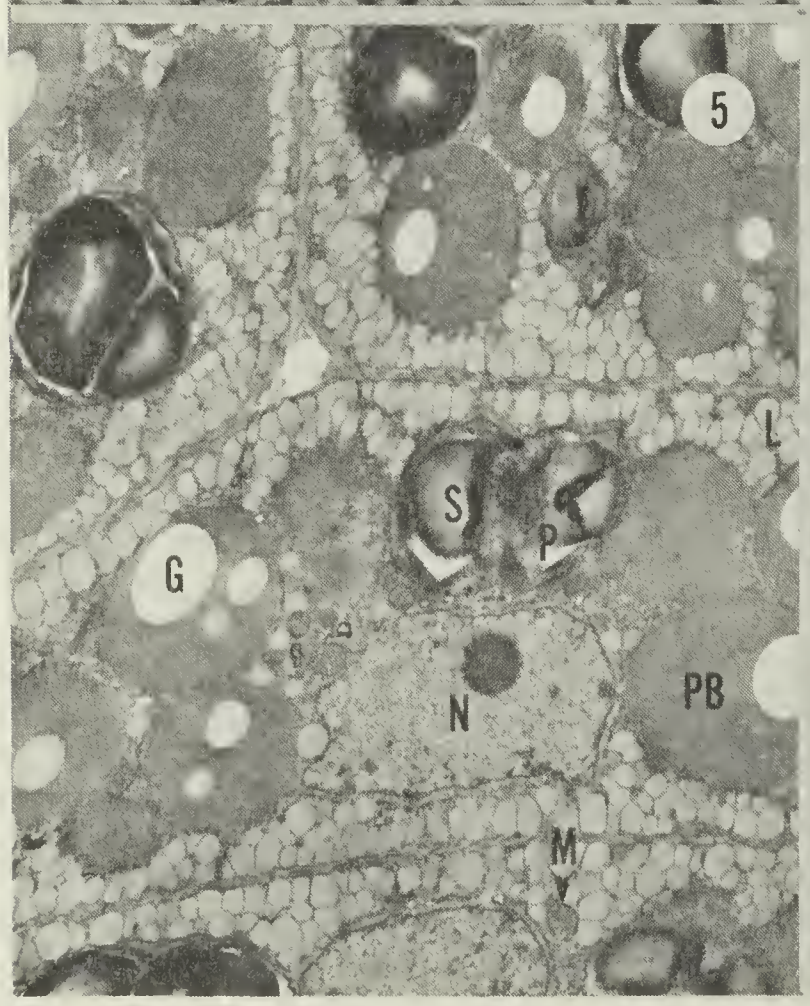
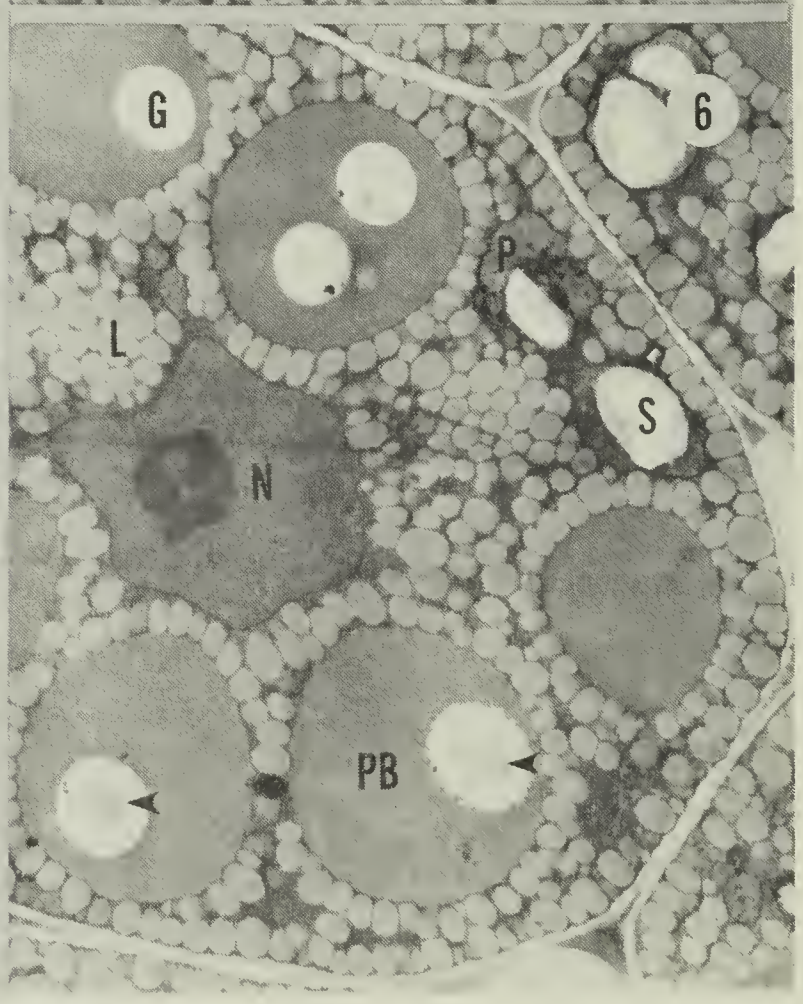
Fig. 1-6. Ultrastructural features of developing seed cotyledon mesophyll cells of *Pharbitis nil*. 1. View of a mesophyll cell 17 days after anthesis. Most of the space within the cell is occupied by vacuoles. X 7,500. 2. View of a plastid showing details of the internal membrane organization (17 days after anthesis). Stacks of thylakoids forming grana are evident (arrow). Carbohydrate in the form of starch and a prolamellar body can also be seen. X 30,000. 3. Mesophyll cells 30 days after anthesis stained with PAS for carbohydrate. Many localized regions of positive staining are present. X 1,350. 4. Mesophyll cells 30 days after anthesis stained with analine blue-black for total protein. Many localized regions of positive staining are present. X 1,350. 5. View of a mesophyll cell 30 days after anthesis. Peripherally-located lipid bodies and centrally-located protein bodies are visible. Globoids are present within the protein bodies. Nuclei and plastids (with starch) are also present. X 7,500. 6. View of a mesophyll cell 40 days after anthesis. Numerous protein bodies, lipid bodies, and plastids are present. Note phytin crystals contained in globoids (arrow). X 7,500.









Key to labeling: V, vacuole; N, nucleus; S, carbohydrate; G, globoid; P, plastid; PB, protein body; PL, prolamellar body; L, lipid; M, mitochondria; D, dictyosome; ER, endoplasmic reticulum.

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Genetic Relationships Between A Southern Population, Formerly *Plethodon Longicrus*, and Three Northern Populations of *Plethodon Yonahlossee*

Mark E. Blaesing and Karen L. Hagen[1]
Department of Surgery
University of Illinois Medical Center
Chicago, Illinois 60612

[1]Present address: Department of Microbiology and Immunology, University of Illinois Medical Center, Chicago, Illinois 60612

ABSTRACT

Polyacrylamide gel electrophoresis was used to study enzyme variation at 13 presumed genetic loci between one population (type locality) of the former *Plethodon longicrus* and three populations of *P. yonahlossee* from the northern half of its range. Nine of 13 loci examined were found to be polymorphic. Estimates of genetic similarity indicate that there are two distinct groups, the former *P. longicrus* population, and the three northern *P. yonahlossee* populations. As other southern or intermediate populations were not sampled, the taxonomy of *P. yonahlossee* cannot be thoroughly analyzed. Electrophoretic data as well as other evidence suggest that the genetic divergence observed is not clinal and thus there probably are at least two groups undergoing speciation and these should be classified as two subspecies, perhaps even as two distinct species.

INTRODUCTION

The taxonomic status of *Plethodon longicrus*, the crevice salamander from Bluerock Mountain, North Carolina has not yet been clearly established. Alder and Dennis (1962) described *P. longicrus* as a species separate from *P. yonahlossee* on the basis of morphological and ecological criteria. Pope (1965) suggested that *P. longicrus* and *P. yonahlossee* may have a subspecific relationship. Later Highton (1972) and Guttman et al. (1978) concluded that these salamanders were conspecific. Guttman et al. (1978) based their conclusions on both biochemical and morphological observations; however, all populations studied were from the southern half of the range of *P. yonahlossee* (southwestern North Carolina and southern Tennessee). Populations of the former *P. longicrus* are known from three

localities (Guttman et al., 1978), all of which occur at the southern end, and are an extension of, the range of *P. yonahlossee*. The two morphs were found to be sympatric at the Bearwallow Mountain, North Carolina, locality by Guttman et al. (1978). Duncan and Highton (1979) studying *Plethodon* of the Ouachita Mountains also examined two northern populations of *P. yonahlossee* using electrophoresis and found virtually no difference between the two populations. At the present time there has been no comparison of the relationship of the former *P. longicrus* to northern *P. yonahlossee*. The purpose of this study is to biochemically analyze the genetic relationships between populations of *P. yonahlossee* from the northern half of its range and the type locality population of the former *P. longicrus* (Bluerock Mountain). Because of limited collecting time, other southern or intermediate populations were not able to be sampled.

Guttman et al. (1978) used primarily starch gel electrophoresis in the biochemical analysis of genetic differences, while we employed polyacrylamide disc gel electrophoresis in the present study. Polyacrylamide gel electrophoresis has been shown to have greater potential for resolving enzyme variations (Hunter et al., 1964; Frederick, 1964; Raymond and Wang, 1960; Ornstein, 1964).

MATERIALS AND METHODS

Salamanders were collected from the following four localities during May, 1979: Iron Mountain, Tennessee; Comers Rock, Virginia (near the northern tip of its range); Mount Jefferson, North Carolina; and Bluerock Mountain, North Carolina. Localities for this study and the study by Guttman et al. (1978) are shown in Fig. 1. Specimens from each locality were collected within a few ha and they were kept alive until their return to the laboratory. Homogenates were prepared from decapitated salamanders, minus stomach contents, and the homogenates were stored below −70 C until analysis. Aliquots of each homogenate were centrifuged at 2000 g for 30 min before electrophoresis. The gels were prepared according to the method described by Hagen et al. (1978) except for some enzymes which required a greater percentage of acrylamide in the gel in order to obtain better resolution. The gels, buffers and stains used are described in Table 1. Staining methods were followed exactly except that PMS was omitted from the ADA stain and α-naphthyl acetate was the only substrate used to detect esterases. Proteins that are known to be controlled by more than one locus are designated with integers ("1" being assigned to the farthest band or set of bands from the origin). Polymorphic proteins controlled by a single locus are designated alphabetically with "a" being assigned to the band migrating farthest from the origin.

All phenotypes were analyzed as if they were the result of genotypes expressed as codominant Mendalian alleles, despite the lack of breeding studies. This assumption has been commonly accepted in other biochemical studies, including those on *Plethodon* by Highton and Webster (1976), Duncan and Highton (1979) and Guttman et al. (1978). Genetic similarities between populations were estimated by computing the genetic similarity, "S" (Rogers, 1972) and the genetic identity for small samples, "I" (Nei, 1978). Estimates of heterozygosity, the mean proportion of loci heterozygous per individual (H) and the mean number of alleles per locus (A) were calculated using direct counts.

RESULTS

Only four of the 13 loci analyzed were found to be monomorphic in all specimens; allelic frequencies for the nine polymorphic loci studied are shown in Table 2. The Bluerock Mountain population (formerly *P. longicrus*) exhibits polymorphic variants that are not expressed in the three northern *P. yonahlosse* populations at the *MR-2* and *Hp* loci, and a polymorphic variant at the *Est-1* locus which is only present in the Comers Rock population at a very low gene frequency (.03). The polymorphic Bluerock Mountain population illustrates complete divergence from the three monomorphic northern populations at the *Hp* locus. Genetic similarity estimates between the populations are shown in Table 3. "I" values between the 3 northern populations range from .96-.97 (Ï = .96), and "S" values are .87 or greater (S̈ = .88) showing a high degree of similarity. In contrast, "I" values between the Bluerock Mountain population and the three northern populations range from .82-.86 (Ï = .84) and "S" values range from .74-.77 (S̈ = .76), indicating considerably less genetic similarity.

Estimates of genetic heterozygosity (H) and the mean number of alleles per locus (A) are given in Table 4. The Bluerock Mountain population has a greater genetic heterozygosity (H = .358) than the three northern populations (range of H = .132-.177, Ḧ = .149). The mean number of alleles per locus is greatest in the Bluerock Mountain population (A = 1.77).

DISCUSSION

The polyacrylamide gel electrophoretic data present evidence that significant genetic divergence has occurred between the Bluerock Mountain population and the three northern populations (Ï = .84), while little divergence has occurred among the three monotypic northern populations (Ï = .96). We were not able to test directly, whether the observed genetic divergence is clinal, as other southern or intermediate populations were not able to be sampled. However, several observations indicate that the observed divergence is probably not clinal. Several topographical features which impose habitat and altitudinal restrictions on *P. yonahlossee* supplemented by direct observation, indicate that the Bluerock Mountain population seems to be genetically isolated from the three northern populations. The Bluerock Mountain population is restricted to an area of approximately 4.1 ha (Alder and Dennis, 1962) and is approximately 4 km from the nearest known population at Grant Mountain. By our own observations we have also observed the Grant Mountain population to be restricted in its range. The Bluerock Mountain and Grant Mountain populations are separated by a valley (about 475 m above sea level) and Reedypatch Creek which may serve as barriers. The habitat restrictions on this salamander along with the distance between known populations would probably not allow significant migration between these isolated populations. Pope (1950) reported that *P. yonahlossee* was not abundant in the southern part of its range, while Hairston (1949) stated that it was rare in the Black Mountains, also near the southern tip of the range. Pope (1950) and Hairston (1949) reported distinct localities in the southern section which were well removed from one another, and gave no indication of a continuous range. Hairston (1949) found 80 percent of his records of *P. yonahlossee* in the Black Mountains from two virgin forest areas, and stated that this species was not distributed throughout the forest but was restricted to an area near streams (within 100 feet). The isolation of the

Bluerock Mountain population from the 3 northern populations and probably most pure *P. yonahlossee* morph populations is enhanced by the Broad River. Finally our field work suggests that *P. yonahlossee* in the southern part of its range exists as a discontinuous distribution of genetic isolate populations.

Electrophoretic data also support the theory that the genetic divergence observed in this study is not clinal. Three loci mentioned earlier, MR-2, Hp and Est-1 possess variants either not present in the Bluerock Mountain population, or not present in the three northern populations. Guttman et al. (1978) found three variants (Idh-1c, Pgm-3a, and Pgm-3b) present in one or more populations of *P. yonahlossee*, from areas farther south than those of this study, which were not present in the three former *P. longicrus* populations (including the Bearwallow Mountain population where both morphs are present). Conversely, Guttman et al. (1978) found two variants (Alb-1c and Alb-2d) which were present in the former *P. longicrus* populations but not in the *P. yonahlossee* populations.

At the present time, assigning taxonomic status to these two distinct groups, based on the observed level of genetic divergence, is speculative. Genetic identities as great as .8-.9 have been observed between sympatric, nonhybridizing species *P. glutinosus* and *P. jordani* (Duncan and Highton, 1979). In contrast Duncan and Highton (1979) described a new species, *P. fourchensis*, having a genetic identity of .75 to its closest relative, *P. ouachitae*, despite the fact these two salamanders hybridized in a narrow area where their ranges came in contact. Highton and Webster (1976), comparing populations of *P. cinereus* from non-glaciated areas, observed genetic identities frequently below .9 (Ï = .87), and suggested these populations might be a group of "semispecies" whose future evolution would be independent from one another.

Based on the level of genetic identity between the Bluerock Mountain population and the three northern populations, the most accurate classification of these two groups is probably to assign each as a different subspecies or race of *P. yonahlossee*. However, there is also the possibility that these two groups are distinct species. What we may be observing at the present time is at least two distinct groups undergoing speciation. Guttman et al. (1978), comparing the three former *P. longicrus* populations to three *P. yonahlossee* populations from the southern part of the range, reported a greater genetic identity, Ï = .95 (Bluerock Mountain population to three southern populations Ï = .92) and a greater genetic similarity, S̈ = .93 (Bluerock Mountain population to three southern populations S̈ = .91) than those observed in this study (Ï = .84, S̈ = .76), and they placed *P. longicrus* in the synonomy of *P. yonahlossee*. The observed differences between these two studies may be the result of a significant genetic divergence of the southern *P. yonahlossee* populations as a whole (including the former *P. longicrus* populations) from the northern populations. Alternatively, the increased resolution of variant proteins by the polyacrylamide gel electrophoresis methods used in the present study may also contribute to our inability to confirm the work of Guttman et al. (1978). Evidence of the increased resolving power of polyacrylamide gel electrophoresis is illustrated by the much greater heterozygosity (H = .358) and mean number of alleles per locus (A = 1.77) for the Bluerock Mountain population in our study than was reported in the study of Guttman et al. (1978) (H = .017 and A = 1.10).

Our estimates of heterozygosity for the three northern populations (Ḧ = .149) is only slightly greater than the mean for amphibians of .079 (Nevo, 1978). The

much greater heterozygosity and number of alleles per locus of the Bluerock Mountain population as compared to the three northern populations might be explained by the older southern population (assuming the three northern populations migrated from the south after the glacial periods) having acquired more polymorphic variants (alleles). Highton and Webster (1976) reported heterozygosity and the total number of alleles to be less in populations of *P. cinereus* from formerly glaciated areas than populations from unglaciated areas, and suggested this was the result of relatively younger northern populations acquiring fewer new traits. The populations from the three northern localities in this study, although from unglaciated areas, may have been influenced more by the glaciers than the more southerly Bluerock Mountain population, as the northern regions may have been uninhabitable during the glacial periods.

The possibility exists that the type locality of *P. yonahlossee* (Grandfather Mountain) is more closely related to the former *P. longicrus* populations, than the three northern populations examined in this study, and therefore the task of assigning nomenclature for these two distinct groups is difficult at the present time. Further studies over the entire range of *P. yonahlossee* are necessary to resolve these important questions about the taxonomy and evolution of these salamanders.

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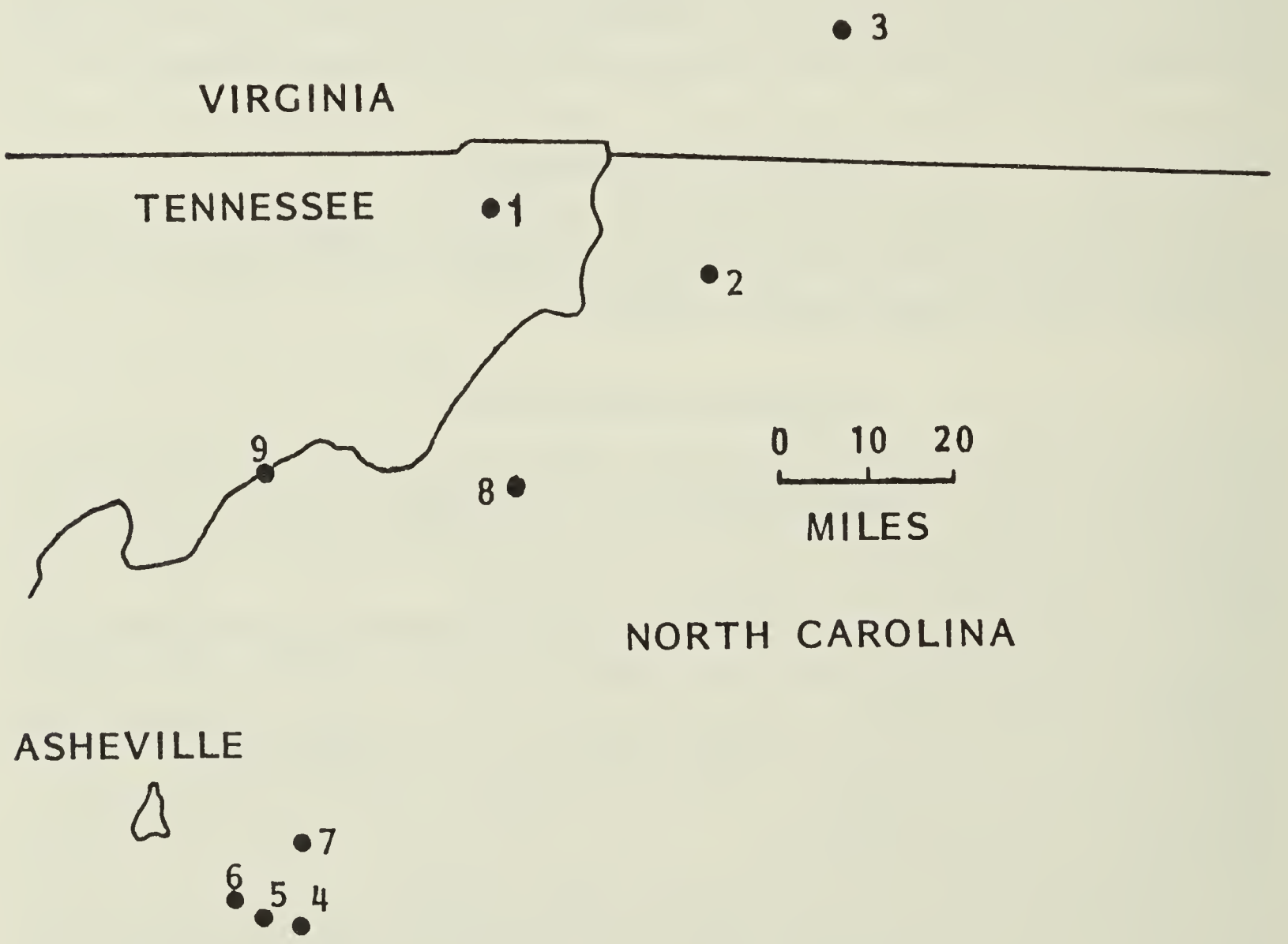

Fig. 1. Relative localities of *P. yonahlossee* for this study (1-4) and the study of Guttman et al., 1978(4-9). 1 = Iron Mountain, 2 = Mount Jefferson, 3 = Comers Rock Picnic Ground, 4 = Bluerock Mountain (type locality of former *P. longicrus*), 5 = Grant Mountain (former *P. longicrus*), 6 = Bearwallow Mountain (both morphs), 7 = Lakey Gap, 8 = Grandfather Mountain, 9 = Crest of Unaka Mountains.

Table 1. Polyacrylamide disc gel electrophoresis methods. a = Tris-glycine, pH 8.9; b = Tris-glycine, pH 8.5; c = Tris-HCL, pH 8.9 (Hagen et al., 1978); d = Barbitol-Tris, pH 7.0; e = Tris-HCL, pH 7.5 (Williams and Reisfeld, 1964); f = Tris-citrate, pH 8.8 (Schiff, 1975).

Protein Tested	Upper Buffer	Lower Buffer	Gel Buffer	Percent Acrylamide	Reference for Stain
Haptoglobin	a	d	c	7.5	Hagen et al., 1978
Esterases	d	d	e	7.5	Van Zutphen, 1974*
Adenosine Deaminase	a	b	f	8.0	Spencer et al., 1968*
Mannose-6 Phosphate Isomerase	a	b	f	10.0	Nichols et al., 1973
Malic Enzyme	a	b	c	7.5	Harris and Hopkinson, 1976
Methemoglobin Reductase	a	b	c	7.5	Niethammer and Huennekens, 1971

*Modified

Table 2. Allelic frequencies of polymorphic enzyme loci observed from populations of *P. yonahlossee* and the former *P. longicrus*.

Locality	1 Iron Mountain	2 Mount Jefferson	3 Comers Rock	4 Bluerock Mountain
Locus (N)				
MPI (5,7,19,9)	a (.60) b (.40)	a (.57) b (.43)	a	a (.67) b (.33)
MR-2 (10,7,18,9)	b	b	b	a (.11) b (.89)
Hp (10,7,19,9)	a	a	a	b (.28) c (.72)
ME-2 (10,7,19,9)	a (.65) b (.35)	a (.93) b (.07)	a (.68) b (.32)	a (.67) b (.33)
Est-1 (10,7,18,8)	b	b	a (.17) b (.81) c (.03)	b (.44) c (.56)
Est-2 (9,5,17,9)	a (.17) b (.83)	a (.70) b (.30)	a (.21) b (.79)	a (.50) b (.50)
Est-3 (10,7,19,9)	a (.80) b (.20)	a (.93) b (.07)	a (.89) b (.11)	a (.72) b (.28)
Est-4 (10,7,19,8)	b (.55) c (.45)	b (.79) c (.21)	a (.29) b (.53) c (.18)	a (.75) b (.06) c (.19)
Est-5 (7,5,6,9)	a (.50) b (.50)	a (.80) b (.20)	a (.75) b (.25)	a (.50) b (.50)

ADA*, MR-1, ME-1 and Hg were monomorphic.
*ADA for location 1 was not tested.

Table 3. Genetic identity (I) and genetic similarity (S) between populations of *P. yonahlossee* and the former *P. longicrus*. The means of group comparisons are also given.

	I				S			
Population	1	2	3	4	1	2	3	4
1 Iron Mountain		.957	.969	.816		.874	.894	.773
2 Mount Jefferson			.956	.832			.868	.740
3 Comers Rock				.863				.770
4 Bluerock								

Groups	Ï	S̈
Within Northern *P. yonahlossee* (Populations 1,2,3)	.961	.879
Between Northern *P. yonahlossee* and Former *P. longicrus* (Populations 4)	.837	.761

Table 4. Observed genetic heterozygosity (H) and mean number of alleles per locus (A) of populations of *P. yonahlossee* and the former *P. longicrus*.

Population	H Observed Mean Proportion of Heterozygous Loci per Individual	A Mean Number of Alleles per Locus
1 Iron Mountain	.177	1.50
2 Mount Jefferson	.137	1.46
3 Comers Rock	.132	1.62
4 Bluerock Mountain	.358	1.77

Ḧ = .149 for the three northern populations (1,2,3)

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Effects of Medina on Corn and Soybean Production

J.H. Jones and F.J. Olsen
Department of Plant and Soil Science
Southern Illinois University
Carbondale, Illinois 62901

ABSTRACT

Several new products have appeared on the market in recent years which are reported to improve soil properties and increase crop yields. One of these products is Medina. A field study was undertaken to determine the effects of varying rates of Medina on soil characteristics and crop production. This research was conducted on a moderately well-drained Hosmer silt loam. The Hosmer series is classified as a fine-silty, mixed, mesic Typic Fragiudalf. Corn (*Zea mays* L.) and soybeans (*Glycine max* L.) were planted in a split-block experimental design. Main treatments were fertility levels (soil applied Medina with no starter fertilizer, soil applied Medina plus starter fertilizer, soil applied Medina plus starter fertilizer and 33 metric tons of manure/ha and foliar applied Medina plus starter fertilizer). Sub-plots consisted of 0, 4.7, 9.4, and 18.8 liters/ha of Medina broadcast sprayed non-incorporated pre-emergent and also a post-emergent application on corn and soybeans. The effects of Median on the soil nutrient status, soil crusting, soil moisture levels, and the populations of soil bacteria, fungi and actinomycetes were evaluated. The influence of Medina on corn and soybean nutrient uptake and yields was also determined. Under the conditions of this study, Medina did not significantly alter the nutrient level of the soil, the nutrient uptake by corn or soybeans, the population of soil microorganisms, soil crusting, or levels of soil moisture, and was ineffectual in improving crop production.

INTRODUCTION

In the early 1950s, considerable research was conducted on synthetic soil conditioners. Since then there has not been much interest in soil conditioners until recently when several new products have appeared on the market which may influence soil properties and crop yields. One of these products is Medina which has been classified under several categories. In some states, it is listed as a soil conditioner, in others as a soil activator, elsewhere as a micronutrient solution. Medina is reported to improve soil physical properties, to increase microbial activity

though it is not an inoculum, and supply micronutrients to improve crop quality and yields. It contains 0.5% magnesium, 0.1% iron, and 0.05% zinc according to the label. It is recommended that this product be soil or plant applied at the rate of 9.4 liters/ha (1 gal/acre). The maximum response reportedly occurs after the second year's application.

The effects of hydrolyzed polyacylonitrile (HPAN) and modified vinyl acetate acid (VAMA) on soil structure were studied by Mortensen and Martin (14). They reported that both chemicals increased the number of water-stable aggregates. Also, there was a significant increase in aeration porosity. Duley (5) evaluated the influence of HPAN on water intake, runoff and erosion on a silty clay loam. The use of HPAN was found to reduce erosion and runoff while increasing infiltration as a direct result of an increase in water-stable aggregation.

Kijne (10) evaluated the effects of VAMA and polyvinyl alcohol (PVA) on water movement and infiltration on a clay loam. Application of both chemicals resulted in a 1.5-fold to 3-fold increase in soil-water diffusivity over a wide range of volumetric water contents. The influence of VAMA on stabilizing surface clods of cropped soils of varying concentrations of clay was investigated by Blavia et al. (1). Clay content was observed to affect the results of VAMA treatments. The higher the clay content the less desirable the result. Laws (11) also determined that the clay content affected the results of VAMA. Contents of 15% clay gave the best results using VAMA on an acid Cecil clay soil.

In laboratory studies using liquid acricultural grade H_3PO_4, Thien (18) concluded that H_3PO_4 liquid significantly increased water stable aggregates. The increase lasted through at least five wetting and drying cycles. Field studies on the longevity of stabilized soil aggregates were carried out by Strickling (17) using VAMA. The effects of VAMA declined with each growing season. This was attributed to freezing and thawing action. Holmes and Toth (8) also reported a decline in stabilized aggregates in field studies with time. They attributed this to mechanical cultivation. Jacobson and Swanson (9) studied the effect of soil types on the duration of response to VAMA. The longest response to the conditioner, 3-4 years, was observed on soils with high silt and clay contents.

Biological activity in the soil in response to VAMA and HPAN was assessed by Fuller and Gairaud (6). VAMA stimulated biological activity, but HPAN had little effect using evolution of CO_2 as a measuring tool. In contrast, Martin and Aldrich (12) found that VAMA decreased bacteria and actinomycete populations while increasing fungi populations. Weaver et al. (19) studied the activities of Medina and Supernate soil conditioners on soil microorganisms. Soil microorganism activity was measured by CO_2 evolution and enumeration using standard dilution and plate count techniques. After 19 days of incubation, no significant CO_2 evolution was reported as a result of the conditioner applications.

The effects of VAMA on crop yields have also been evaluated (3, 6, 9). VAMA increased corn (*Zea mays* L.) yields but had no effect on soybean (*Glycine max.* L.) yields. In a greenhouse study, Doyle and Hamlyn (4) reported that VAMA increased tomato (*Lycopersicon esculentum* Mill.) yields in direct relation to the increase of water-stable aggregates formed. The findings of Holmes and Toth (8) indicated that increases in yield of field crops were due to changes in soil physical properties by VAMA. The VAMA created a dust mulch which effectively reduced water loss by evaporation and thus more water was available to the crop. Another benefit was a reduction in the severity of soil crusting, thereby allowing greater

crop emergence. DeMent et al. (3) also reported a decrease in soil crusting and an increase in crop emergence. Although researchers have reported significant yield increases from soil conditioners, Haise et al. (7) noted that a decrease in sugar beet (*Beta vulgaris* L.) yields using VAMA and HPAN.

Overdahl (15) studied the effects of Medina, Sol-Ez, and Bayfolan on corn production over a 2-year period. He concluded that there was no increased corn yields from the use of these materials. Roth (16) reported slight but not significant yield increases in corn, soybeans, and grain sorghum [*Sorghum bicolor* (L.) Moench] production using Medina.

The objectives of this study were to determine the effects of varying rates of Medina on soil characteristics and crop production.

MATERIALS AND METHODS

The research was conducted over a 3-year period (1978-1980) at the west unit of the Agronomy Research Center, Southern Illinois University, Carbondale, Illinois. The experimental site was a moderately well-drained Hosmer silt loam. The Hosmer series is classified as a fine-silty, mixed, mesic Typic Fragiudalf. The soil was limed and fertilized to soil test requirements prior to the initiation of the study.

Corn (*Zea mays* L. cv. 'Pioneer 3184') and soybeans (*Glycine max* L. cv. 'Williams') were planted in a split-block experimental design with four replications. The corn main plots were 18 by 15 m and the subplots were 4.5 by 15 m. The soybean main plots were 18 by 7.5 m and the subplots were 4.5 by 7.5 m.

Main treatments were fertility levels (soil applied Medina with no starter fertilizer, soil applied Medina plus starter fertilizer) [135 kg/ha of 4-24-24], soil applied Medina plus starter fertilizer and 33 metric tons/ha of manure, and foliar applied Medina plus starter fertilizer). The subplot treatments consisted of 0, 4.7, 9.4, and 18.8 liters/ha of Medina broadcast sprayed non-incorporated preemergent and also a post-emergent application at the 5-leaf stage for corn and at the 5th trifoliate stage for the soybeans. All plots received herbicides in addition to hand weeding for weed control. Irrigation was used the first year when conditions warranted. The elemental contents of corn and soybean plant tissue were analyzed. Grain yields were determined for both crops.

A pocket penetrometer was used to assess the force required in kg/cm^2 to break through the soil crust. The gravimetric determination of the soil moisture percentages was made periodically during the growing season from the A_p and B horizons. Disturbed soil samples were used to evaluate the amounts of plant available moisture. The pressure plate apparatus was used to find the percent moisture held by the soil at tensions of 100 cm, 1/3 atm, and 15 atms corresponding to aeration porosity, field capacity, and permanent wilting percentage respectively. Soil samples taken to a depth of 18 cm were used for soil chemical analyses.

Microbial populations in the top 7.5 cm of soil were enumerated using the plate count technique. Populations of soil bacteria, fungi and actinomycetes were estimated by plate counts using the following media cultures, respectively: soil extract agar, glucose-peptone acid agar (2), and Lingappa Lockwood chitin agar (13).

RESULTS AND DISCUSSION

The value of a soil conditioner, soil activator, or a micronutrient solution lies in their ability to increase crop yields under varying conditions. Soil levels of

primary and secondary nutrients in the corn trials treated with varying rates of Medina without starter fertilizer are shown in Tables 1 and 2. There were no significant increases in any of the primary and secondary nutrients in the soil at the end of the second growing season as a result of applying Medina except for iron in the 9.4 liter/ha treatment.

Leaf tissue analyses of corn were determined in 1978 and 1979 (Tables 3 and 4). The different Medina rates used had no influence on the nutrient uptake by the corn plants. Plant response from Medina in the form of increased biomass production was not observed.

Soil populations of actinomycetes, bacteria, and fungi in the corn experiment in 1979 were not increased with Medina (Table 5). This data suggests that Medina applied at the recommended rates would not enhance microorganism populations in the soil.

Soil crusting measured by the penetrometer and soil moisture levels determined periodically during the growing season were used to evaluate the value of Medina as a soil conditioner. Surface soil crusting and average moisture percentages for the A_p and B soil horizons showed no differences with the varying rates of Medina application (Tables 6 and 7).

Any positive changes attributable to Medina influencing soil microorganisms, soil physical and chemical properties, and/or nutrient uptake by crop plants should be reflected in increased crop yields. The mean corn and soybean yields on four soil treatments with different levels of Medina application over three production years are shown in Tables 8 and 9. Although corn and soybean yields varied from year to year due to the amount and distribution of rainfall during the growing season, the application of Medina had no significant affect on crop yields for any of the three production years.

Under the conditions of this study, the applications of Medina did not significantly alter the nutrient level in the soil, nutrient uptake by the crop plants, soil microorganism populations, soil crusting, soil moisture levels, or the yields of corn or soybeans during a 3-year study.

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Table 1. Soil Levels of Selected Primary and Secondary Nutrients in Corn Plots Without Starter Fertilizer (September 1979).

	P		K	Ca	Mg	S
Medina Rate	Bray 1	Bray 2				
liters/ha	ppm					
0	14 a*	24 ab	130 a	812 a	40.0 a	9.5 a
9.4	13 a	21 a	139 a	1,037 a	40.0 a	9.5 a
18.8	15 a	26 b	140 a	1,062 a	39.9 a	8.7 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 2. Soil Levels of Selected Micronutrients in Corn Plots Without Starter Fertilizer (September 1979).

Medina Rate	Soil ph 1:1 (soil:water)	Zn	Mn	Fe	Cu	B
liters/ha		ppm				
0	7.01	2.5 a*	63 a	30 a	2.9 a	0.40 a
9.4	7.03	2.4 a	58 a	25 b	2.4 a	0.45 a
18.8	7.10	2.8 a	66 a	29 a	2.8 a	0.47 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 3. Leaf Tissue Analyses of Corn for the 1978 and 1979 Growing Seasons.

Main Treatment	Medina Rate	N	S	P	K	Mg	Ca	Na
	liter/ha			1978 %				
	0	--	--	.29 a*	1.98 a	.23 a	.48 a	.24 a
	9.4	--	--	.30 a	2.08 a	.22 a	.47 a	.02 a
	18.8	--	--	.29 a	1.98 a	.24 a	.50 a	.01 a
Soil Applied Medina				1979				
	0	2.24 a	.18 a	.24 a	1.82 a	.17 a	.48 a	.01 a
	9.4	2.35 a	.18 a	.26 a	2.15 a	.16 a	.48 a	.01 a
	18.8	2.32 a	.18 a	.26 a	2.20 a	.16 a	.48 a	.01 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 4. Leaf Tissue Analyses of Corn for the 1978 and 1979 Growing Seasons.

Main Treatment	Medina Rate	Fe	Al	Mn	B	Cu	Zn
	liters/ha			1978 %			
	0	143.5 a*	69.8 a	71.3 a	19.3 a	10.5 a	24.0 a
	9.4	117.3 a	72.5 a	71.0 a	17.8 a	10.0 a	26.0 a
	18.8	107.5 a	65.0 a	71.8 a	16.8 a	10.0 a	21.5 a
Soil Applied Medina				1979			
	0	118.0 a	50.0 a	50.8 a	7.8 a	12.0 a	20.0 a
	9.4	125.0 a	50.5 a	55.3 a	9.3 a	12.8 a	28.5 a
	18.8	117.3 a	42.5 a	49.0 a	9.0 a	12.5 a	19.8 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 5. Enumeration of Soil Populations of Actinomycetes, Bacteria, and Fungi in the Corn Experiment During 1979.

When Sampled	Medina Rate	Microorganisms per Gram of Oven Dry Soil		
		Actinomycetes (x 10^5)	Bacteria (x 10^7)	Fungi (x 10^3)
	liters/ha			
Prior to application of Medina, 5/12/79	0.0	27.0 a*	30.6 a	37.9 a
7 days after application of Medina	0.0	48.0 a	197 a	26.2 a
	9.4	56.8 a	184 a	29.2 a
	18.8	46.2 a	182 a	27.6 a
14 days after application of Medina	0.0	36.0 a	177 a	30.3 a
	9.4	57.4 a	179 a	30.6 a
	18.8	40.7 a	158 a	31.8 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 6. Evaluation of Surface Soil Crusting by Penetrometer in the Corn Experiment During the 1978 and 1979 Growing Seasons.

Date	Medina Rate	Force Required to Break Soil Crust
	liters/ha	----------kg/cm^2----------
10/1/78	0	0.78 a*
	9.4	0.75 a
10/6/79	0	1.83 a
	9.4	1.82 a
	18.4	1.71 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 7. Averages of Moisture Percentages in Soil Horizons Taken in the Corn Experiment During the 1979 Growing Season.

Date	Medina Rate	Percent Moisture by Weight Horizon A_p	B
	liters/ha		
	0	12.8 a*	12.0 a
7/30/79	9.4	12.5 a	11.0 a
	18.8	14.7 a	11.5 a
	0	12.5 a	11.5 a
10/4/79	9.4	12.5 a	12.1 a
	18.8	13.7 a	10.5 a

*Means followed by the same letter in each column are not significantly different at the 5% probability level according to Duncan's Multiple Range Test.

Table 8. Mean Yields of Corn Harvested During the 1978-80 Growing Seasons.

Main Treatment	Medina Rate	Corn yields at 15.5% moisture 1978	1979	1980
	liters/ha	kg/ha		
Soil applied	0	4,433 a*	9,723 a	1,693 a
Medina	4.7	4,038 a	10,253 a	2,312 a
	9.4	3,724 a	9,853 a	1,769 a
	18.8	3,511 a	10,294 a	1,521 a
Soil applied	0	5,549 a	11,145 a	1,981 a
Medina + starter	4.7	4,050 a	11,312 a	2,203 a
fertilizer	9.4	4.828 a	10,924 a	1,932 a
	18.8	4,514 a	10,716 a	1,873 a
Soil applied	0	4,891 a	11,150 a	1,710 a
Medina + starter	4.7	5,079 a	11,476 a	1,330 a
fertilizer + 1978	9.4	4,853 a	11,195 a	2,194 a
applied manure	18.8	4,402 a	10,974 a	1,998 a
Foliar applied	0	5,267 a	11,105 a	1,602 a
Medina + starter	4.7	5,392 a	9,908 a	1,615 a
fertilizer	9.4	5,292 a	10,676 a	2,029 a
	18.8	4,690 a	10,423 a	1,373 a

*Means in the same column with the same main treatment followed by the same letter are not significantly different at the 5% level of probability.

Table 9. Mean Yields of Soybeans Harvested During the 1978-80 Growing Seasons.

Main Treatment	Medina Rate	Soybean yields at 13% moisture 1978	1979	1980
	liters/ha	kg/ha		
Soil applied Medina	0	1,626 a*	2,562 a	1,456 a
	4.7	1,781 a	2,665 a	1,591 a
	9.4	1,532 a	2,722 a	1,724 a
	18.8	1,626 a	2,941 a	1,711 a
Soil applied Medina + starter fertilizer	0	1,727 a	2,547 a	1,266 a
	4.7	1,908 a	2,260 a	1,134 a
	9.4	1,875 a	3,014 a	1,266 a
	18.8	1,895 a	2,528 a	1,240 a
Soil applied Medina + starter fertilizer + applied manure	0	1,747 a	2.905 a	1,424 a
	4.7	1,653 a	3,049 a	1,222 a
	9.4	1,747 a	2,911 a	1,438 a
	18.8	1,512 a	3,043 a	1,372 a
Foliar applied Medina + starter fertilizer	0	1,895 a	2,609 a	1,334 a
	4.7	1,922 a	2,445 a	1,307 a
	9.4	2,083 a	2,999 a	1,258 a
	18.8	1,720 a	2,611 a	1,430 a

*Means in the same column with the same main treatment with the same letter are not significantly different at the 5% level of probability.

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Water Solubilization of North Dakota Lignite Via Reductive Carboxylation

Robert C. Duty and Keith Foster
Chemistry Department
Illinois State University
Normal, IL 61761

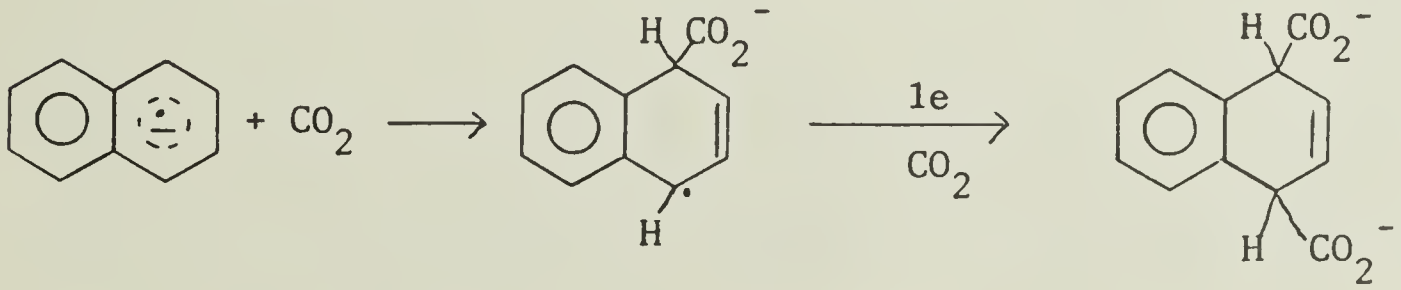
ABSTRACT

The reductive carboxylation of North Dakota lignite was accomplished using an autoclave to react lignite with potassium metal under various temperature and solvent systems. The characterization of the alkali-soluble acids generated and the insoluble residue was accomplished by several titrometric techniques as well as elemental analyses.

INTRODUCTION

The solubilization of coals in alkaline aqueous solutions has been reported for the high pressure reaction of Illinois Bituminous #6 coal with sodium metal in the presence of carbon dioxide. Such solubilization was attributed to the substitution of carboxyl ($-CO_2H$) groups onto the various aromatic structures which comprise coal (Duty, 1981). These carboxyl groups may be substituted onto aromatic rings through two main pathways — direct reaction and Kolbe-type reactions.

The use of alkali metals such as sodium or potassium for the generation of radical anions in aromatic systems has been known for many years. Wawzonek showed that these radical-anions react with carbon dioxide in aprotic solvents to produce a carboxylated aromatic structure by the production of 1,4-dicarboxy-1,4-dihydro-naphthalene from the naphthalenide anion (Wawzonek, 1959) as shown below:

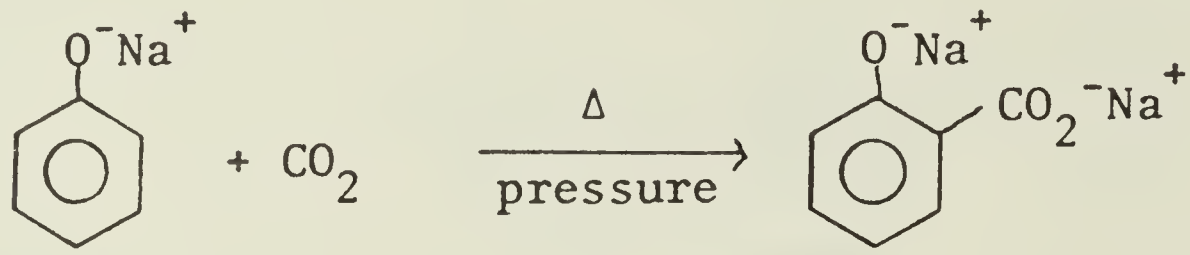


(1)

Moreover, Sternberg and Delle Donne have found that such anions can be produced in coal by reduction of the coal with potassium metal in tetrahydrofuran (THF)

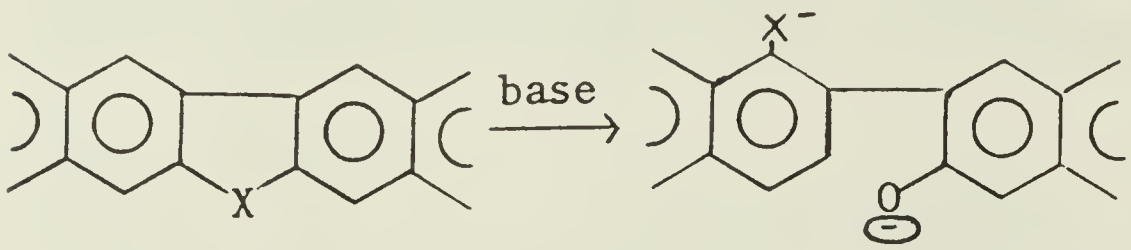
using naphthalene as an electron-transfer agent. These anions were alkylated by treatment with ethyl iodide (Sternberg, 1974). Therefore, upon generation of such radical anions in coal, it seems likely that direct reaction of these with carbon dioxide is likely to occur.

The Kolbe reaction, which was reviewed by Lindsey (Lindsey, 1957), has been known for over 100 years is shown below. Such reactions involve substitution by a carboxyl group ortho to an existing phenolic group as follows:



(2)

Such phenolic groups have been shown to occur in many types of coal, including North Dakota lignite (Blom, 1957). Furthermore, such sites may be formed by cleaving various ether linkages present in coal with alkali metals. These reactions have been experimentally substantiated by Sternberg, *et al.* (1974). Phenolic groups may also be formed by cleaving dibenzothiophene and dibenzofuran structures which are also found in coals (Hayatsu, 1975) as follows:



(3)

X = S or O

Whether by direct reaction with carbon dioxide or through phenolic intermediates or both, North Dakota lignite can be expected to generate a substantial amount of water-soluble acids.

MATERIALS AND METHODS

Reduction and Carboxylation of North Dakota Lignite

Ten grams of solvent-refined North Dakota lignite (200 — 250 US mesh) was dried at 100°C for 2 hours. Approximately 10 g of potassium metal was cut and weighed in a drybox under a nitrogen atmosphere desiccated by P_2O_5. In the solvents toluene and tetrahydrofuran, 10.000 g of biphenyl was added as the electron transfer agent. The biphenyl and coal were placed in an Autoclave Engineers Model WP-5000 autoclave equipped with a 900 rpm steel paddle stirrer. The potassium was covered with solvent and transferred to the autoclave. Table 1 summarizes reaction conditions including solvent, temperature, gases employed, and length of reaction. The autoclave was sealed and purged with nitrogen gas. Reactions 3-5 were not purged with nitrogen but were purged and pressurized immediately with carbon dioxide. The thermocouple which controlled the temperature was inserted into the autoclave body. After the reaction was complete the autoclave was cooled and the contents removed. Isopropyl alcohol (500 ml) was added to the autoclave to decompose any unreacted potassium, and the mixture was stirred for approximately 30 minutes. The contents were removed and this solution was rotary evaporated to remove solvents. The autoclave was washed

also with 5% aqueous potassium hydroxide. This was saved for later use as the basic reflux solvent for solubilizing the black acids.

Separation of Biphenyl and Neutral Compounds from Lignite

The dried autoclave contents were refluxed for 3-6 hours with 750 ml of a 3:1 (v/v) mixture of ether/benzene. The insoluble materials from this reflux were separated by centrifugation using a Sorvall SS-3 Model GLC-1 high-speed centrifuge (10,000 rpm). These were dried and weighed. The ether/benzene solution was collected and the volume was adjusted to 1.000 l. Ten ml of this was taken for HPLC analysis of recovered biphenyl. The remainder was rotary evaporated, dried, and weighed to determine the amount of neutral compounds.

Separation of Acids from Water-Insoluble Residue

The ether/benzene insolubles were placed in a 1.00 l boiling flask and refluxed for 3-6 hours with the 5% KOH solution (0.500 l) which was used to wash the autoclave. The insoluble material remaining was isolated by high-speed centrifugation, dried, and weighed. The supernatant solution was acidified by Congo Red with concentrated HCl. Acids, which precipitated slowly from the acidified solution, were collected by centrifugation, washed several times with distilled water, dried, and weighed. The pH of the final supernatant was adjusted to 7.0 using potassium carbonate. It was extracted with three-200 ml portions of ether, which were dried with $MgSO_4$. The ether was evaporated, leaving amphoteric compounds which were weighed.

Equivalent Weight Determination of Black Acids

Approximately 1 N NaOH was standardized by pH titration with 1.000 N HCl using a Sargent Welch Model HG Automatic Titrator. About 0.25 g of acid sample was weighed to within ± 0.0001 g and dissolved in 10.00 ml of the standardized 1.000 N NaOH solution. This was degased for one hour with N_2 in a vessel previously described (Duty, 1978) and titrated with the 1.000 N HCl. From the difference between the blank NaOH curve and those of the acids in NaOH, the equivalent weight of the acid sample could be determined. The accuracy of the method was determined by finding the equivalent weight of 1,2,4-benzene-tricarboxylic acid, which has an equivalent weight of 70.

Total Acidity and Carboxyl Group Tests

The total acidity of each water-insoluble residue was determined as outlined by Schafer (1970). This was accomplished by stirring the sample (about 0.250 g) in a solution 0.200 N in barium hydroxide and 0.800 N in barium chloride. The residue was separated from the supernatant by filtration under nitrogen, and the residue was washed with a solution 0.1 N in barium chloride and 0.03 N in sodium hydroxide. After washing with distilled water, the residue was boiled for twenty minutes in 20.00 ml of 0.495 N perchloric acid. After filtration, the residue was discarded and the filtrate was diluted exactly to 250.0 ml in a volumetric flask. Twenty ml of this solution was back-titrated using 0.108 N barium hydroxide connected to the Sargent-Welch titrator previously described. All handling of solutions was carried out under a blanket of nitrogen. From the titration data, total acidity as meq/g was calculated.

Carboxyl group determinations were conducted according to the procedures established by Schafer (1970). About 0.25 g of sample was weighed and placed into a 250 ml boiling flask into which was added 100 ml of a 1 N solution of barium acetate. The pH of this solution was adjusted to 8.25 using 0.108 N barium

hydroxide. After 4 hours of refluxing under nitrogen, the solutions were titrated back to the endpoint of 8.25 using the titrator and base previously used. From the titration data, carboxyl groups per gram were determined. Phenolic group content was obtained by the difference between total acidity and carboxyl group values for each sample.

Elemental Analysis of Water-Insoluble Residues and Black Acids

The ash content of each sample was determined by accurately weighing about 0.2 g of the sample into previously dried and weighed crucibles. These samples were ashed in a Thelmco Model GRP muffle furnace at 850°C for 4-6 hours. Percent ash was calculated by weighing the crucible after ashing and finding the weight of ash.

The elemental analyses of acids and residues were conducted by the Micro Analysis Corporation of Wilmington, Delaware. Percent (weight) carbon, hydrogen, nitrogen, and sulfur were obtained. The sum of these plus the ash content were assumed to be the total elemental content except for oxygen. Percent oxygen was obtained by subtracting this sum from 100%.

RESULTS AND DISCUSSION

The overall extent of solubilization in the various reactions was expressed as percent solubility. This quantity was defined as the weight percent of the original lignite for which the insoluble residue did not account. These values are given as well as the material balances of the five reactions in Table 2.

The variations encountered with lignite as a substrate under the reaction conditions are well illustrated by the range of percent solubilities seen in Reactions 3-5. Also, the choice of solvent plays a minor role in percent solubility. In reaction 1, which was carried out in tetrahydrofuran (THF), the percent solubility was 57.8%. In reaction 2, the lignite showed the highest solubility, but this probably was due to the increase in temperature. Chiri (1981) reported an increase in percent solubility of 18.9% from 100°C to 200°C for bituminous coal (Ill. #6) in the solvent toluene. Reactions 3-5 gave a mean percent solubility of 45.1 ± 2.9% and were carried out in toluene (Lavin, 1982).

The greatest amount of direct evidence for carboxylation is the actual isolation of black acids from aqueous alkali extract of the reactions. Furthermore, the material balances all show an increase in weight of coal products from the original starting lignite. The primary reason for this weight gain is uptake of CO_2 and the subsequent incorporation of this into carboxyl groups. Most evidence for the extent of carboxylation was obtained by indirect methods such as titrometric and elemental analysis measurements. The extent of carboxylation is indicated by the increase of the carboxyl groups in the insoluble residue over the starting lignite. The total acidity and carboxyl group determinations were obtained for each by the difference of total acidity and carboxyl group values.

The marked loss in phenolic group content from unreacted lignite to insoluble residue is due to the conversion of these groups to phenolic acids (reaction 2) which are generated during the course of the reaction. Evidence for these phenolic acids is illustrated in Table 2 where the carboxyl group content increases over unreacted lignite and the phenolic content decreases.

Further evidence of carbon dioxide incorporation in lignite structures is the

increase in percent oxygen in both residue and acid when compared to starting lignite. The elemental analysis data for the acids and residues of reactions 1-5 are given in Table 4. Increases in percent oxygen are seen in all acids and residues, indicating that new oxygen-containing functional groups have been added to the coal structure. Rather interesting is the fact that the acids generated from Reactions 1 and 2, which yielded the greatest percent solubility, had the lowest percent oxygen. Reasons for this will be offered later in this report.

From elemental analysis, carboxyl group, and phenolic group data from the insoluble residues, the number of carboxyl and phenolic groups per 1000 carbon atoms was calculated as well as the atomic H/C and O/C ratios. These are presented in Table 5.

These triplicate runs showed remarkable similarities even though the overall percent solubility of each reaction varied from the mean by as much as five percent. (Errors are inherent in this kind of research due to the variable nature of the lignite substrate.) The extent of aromatization in these residues may be compared to that of the unreacted lignite by comparing H/C atomic ratios. Since the H/C ratio of demineralized moisture free (*dmmf*) lignite is considerably lower, one may conclude that the polymeric aromatic structures of the lignite are somewhat disrupted by the carboxylation process (see reaction 1). Therefore, the extent of disruption may very well control the extent of solubilization.

Equivalent Weight of Black Acids

The equivalent weights of the black acids were determined by the titration method described previously. These are shown in Table 6. These data indicate that an inverse relationship generally exists between percent solubility and the equivalent weight of the acids generated. In this reaction, the equivalent weight is a measure of how extensively a substrate is carboxylated. The lower the equivalent weight, the more carboxyl groups are added per unit weight. However, considering the data in Table 4, the two reactions generating acids of the lowest equivalent weight also generated acids having the lowest percent oxygen. This may seem paradoxical, but if one computes percent oxygen in one equivalent of acid due to the carboxyl group and subtracts this from total percent oxygen in each acid, one obtains a percent oxygen contribution from other functional groups. The majority of these other groups are phenolic groups. Table 7 shows these results.

The percent oxygen from other functional groups shows an inverse relationship with percent solubility without exception when the data in Tables 2 and 7 are compared. Since the percent oxygen from other groups is mainly comprised of phenolic group contributions, this may be a measure of "phenolic efficiency" which is solvent dependent. Since there are two paths available for carboxylation to occur (reactions 1 & 2), the data from Table 7 suggest that carboxylation occurs more readily on aromatic carbanions (reaction 1) and, consequently, reflects a lower percent oxygen content. In the toluene solvent, the percent oxygen content suggests carboxylation occurred more on the phenolic sites (reaction 2) than the aromatic sites which is reflected in a higher oxygen content "from other groups" as revealed in Table 7.

One observation made from Table 4 was that percent sulfur markedly decreased in both acids and residues with respect to the starting lignite. This was evidenced macroscopically by the detection of H_2S upon opening the autoclave following the reactions.

An additional observation from Table 4 is that the percent ash in the residue is much higher — nearly five times — than that in the acids. This indicates that the formation of acids indeed is a result of extensive disruption of the aromatic superstructure which is characteristic of lignite. This leads one to conclude that the structure of the insoluble residue is that of a core containing trapped insoluble inorganic compounds surrounded by a carboxylated surface.

SUMMARY

The carboxylation of North Dakota lignite was conducted under a variety of reaction temperatures. The greatest solubility was obtained by using naphthalene as molten solvent and electron transfer agent at 200°C. Triplicate reactions were conducted in toluene, and the percent solubility varied from the mean by about 5%. Total acidity, carboxyl group, and phenolic group determinations were made for all insoluble residues. These yielded insight into the intrinsic extent of carboxylation. Elemental analysis data pertaining to residues and acids were obtained as were the equivalent weights of the acids. From this, it seemed that, as equivalent weight decreased, percent oxygen actually decreased. From a variety of calculations, it was seen that this was actually a measure of how many phenolic groups per unit weight are needed to induce the substitution of a carboxyl group. From decreases in percent sulfur from lignite to products as well as a very large difference in percent ash in acids compared to residue gave insight as to the structures of these moieties.

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Table 1. Autoclave Reactions

Reaction #	Solvent	Temperature	Gas(es) Employed
1	THF	100°C	N_2 (5 days) CO_2 (2 days)
2	Naphthalene	200°C	N_2 (5 days) CO_2 (2 days)
3	Toluene	100°C	CO_2 (5 days)
4	Toluene	100°C	CO_2 (5 days)
5	Toluene	100°C	CO_2 (5 days)

Table 2. Percent Solubility and Material Balance

Reaction No. and Solvent	Temp (°C)	Wt. Insoluble Residue (g)	Wt. of black acids	Total Wt. of All Fractions	% Solubility
1 (THF)[a]	100	4.219	3.78	11.642	57.8
2 (Naph)[a]	200	3.238	4.80	b	67.8
3 ⌀ CH_3	100	5.913	5.11	13.899	40.8
4 ⌀ CH_3	100	5.091	4.43	12.376	49.1
5 ⌀ CH_3	100	5.496	4.46	13.015	45.4
lignite[c]	100	0.807	0.306	1.113	19.3
lignite[d]	100	0.893	0.268	1.161	10.7

[a]10 g of lignite and 10 g of potassium.
[b]The material balance for the second reaction is obtainable since the neutral fraction contained the solvent naphthalene which could not be separated from the neutral compounds.
[c]1 g. of lignite and 50 ml toluene
[d]1 g. of lignite, 1 g. of potassium and 50 ml toluene

Table 3. Total Acidity and Carboyxl Group Data for Insoluble Residues

Reaction	Total Acidity[a] (meq/g)	Carboxyl Groups[a] (meq/g)	Phenolic Groups[d] (meq/g)
1	4.59 ± 0.04	1.80 ± 0.02	2.79
2	5.10 ± 0.12	1.90 ± 0.03	3.20
3	4.64 ± 0.18	1.61 ± 0.03	3.03
4	4.59 ± 0.04	1.55 ± 0.06	3.04
5	4.78 ± 0.02	1.65 ± 0.07	3.31
Unreacted Lignite	6.11[b]	0.78[c]	5.32

[a]average ± average deviation - two determinations
[b]average of three determinations (Lavin, 1982)
[c]average of eight determinations (Lavin, 1982)
[d]obtained by difference

Table 4. Elemental Analysis Data for Reaction Products

	Reaction	%C	%H	%N	%S	%Ash[a]	%0[b]
1	Acids:	66.16	4.45	1.021	0.52	0.986	26.86
	Residue:	64.78	3.49	0.56	0.28	4.63	16.26
2	Acids:	66.09	4.13	0.991	0.58	0.906	27.30
	Residue:	66.31	3.44	0.58	0.33	4.56	24.78
3	Acids:	56.75	4.29	1.021	0.32	0.935	36.68
	Residue:	59.90	4.40	0.929	0.21	4.76	30.73
4	Acids:	63.45	3.81	1.233	0.59	0.772	30.15
	Residue:	60.06	4.01	0.922	0.57	4.84	29.60
5	Acids:	59.48	4.84	1.076	0.51	0.811	33.28
	Residue:	60.34	5.12	0.710	0.48	4.83	28.52
	Unreacted Lignite:	65.71	3.77	0.78	0.89	8.95	19.90

[a]Average of two determinations
[b]By Difference

Table 5. Carboxyl and Phenolic Groups per 1000 Carbon Atoms for Residues

Reaction	Carboxyl Groups/1000 C	Phenolic Groups/1000 C	H/C	O/C
3	32.3	60.7	0.88	0.38
4	31.0	60.5	0.76	0.37
5	32.8	62.2	1.02	0.35
Unreacted Lignite	14.2	97.1	0.69	0.30

Table 6. Equivalent Weight of Black Acids

Reaction	Equivalent Weight (g)[a]
1	226 ± 3
2	215 ± 3
3	267 ± 2
4	259 ± 6
5	257 ± 3
1,3,5-benzenetricarboxylic acid	71.9 ± 2.2

[a]sample size was 0.1000 g of acid

Table 7. Percent Oxygen from Various Functional Groups

Reaction	% 0 from Carboxyl Groups	% 0 from Other Groups
1	14.16	12.70
2	14.88	12.40
3	11.99	24.69
4	12.36	17.79
5	12.45	20.83

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Susceptibility of Chickens to *Campylobacter fetus* subsp. *jejuni*

Thomas E. McQuistion*
Department of Biology
Millikin University
Decatur, Illinois 62522
Cheryl A. Helsing
School of Veterinary Medicine
University of Illinois
Urbana, Illinois 61801
Joseph Sutton
School of Medicine
University of Southern Illinois
Carbondale, Illinois 62901

*Please direct all correspondence to T.E. McQuistion. Telephone Number: (217) 424-6236.

ABSTRACT

Poultry has been implicated in the transmission of *Campylobacter* organisms to humans. To determine the susceptibility of chickens to *Campylobacter* organisms, *C. fetus* subsp. *jejuni* isolated from human patients was inoculated intraperitoneally, intravenously or orally into chickens. *C. fetus* subsp. *jejuni* infections developed in chickens by all three routes of administration. Antibiotics routinely and continuously used as poultry feed additives to control protozoan parasites and enhance feed efficiency were tested for bactericidal action to *C. fetus* subsp. *jejuni*. Salinomycin and monensin, two widely used antibiotics, had no activity against *C. fetus* subsp. *jejuni*.

INTRODUCTION

Campylobacter fetus subsp. *jejuni* has been recently recognized as a causative agent of human gastroenteritis. The development of selective culture techniques designed to isolate *Campylobacter* species from stool specimens has demonstrated

that *Campylobacter* is distributed worldwide and is commonly associated with diarrheal patients as is *Salmonella* or *Shigella* (Karmali and Fleming, 1979).

These discoveries have prompted much speculation and experimentation on the mode of transmission of *Campylobacter fetus* subsp. *jejuni*. Poultry has been implicated in the transmission of *Campylobacter* organisms (Butzler, et al., 1977; Grant, et al., 1980; Leuchtefeld, et al., 1980; Park, et al., 1981; Smibert, 1969; and Smith and Muldoon, 1974). Experimental *C. fetus* subsp. *jejuni* inoculations in poultry have produced varied results. Ruiz-Palacios *et al.* (1981) used 3-day-old chickens inoculated orally with human isolates of *C. fetus* subsp. *jejuni* to demonstrate the invasive power of the bacterium and the pathology of the intestinal mucosa resulting in diarrhea. Manninen et al. (1982), however, reported no deaths or diarrhea in 3-day-old chickens inoculated orally with isolates of *C. fetus* subsp. *jejuni*. The use of mammalian hosts for intestinal colonization of *C. fetus* subsp. *jejuni*, such as mice, rats and rabbits, has had mixed success (Field et al., 1981 and Manninen, et al., 1982). Experimental infections of *C. fetus* subsp. *jejuni* in animals by various methods of inoculation has not been documented.

Virtually all commercial broiler operations routinely use antibiotics in the feed to prevent various protozoan diseases. These antiprotozoan antibiotics such as salinomycin and monensin are designed for continuous use in the feed for virtually the entire life of birds grown specifically for meat production. In addition to their antiprotozoan properties, salinomycin and monensin are bactericidal against certain procaryotes (Miyazaki, et al., 1974; Agtarap and Chamberlin, 1968). If these antibiotics are bactericidal against *Campylobacter* organisms in the intestinal tract of chickens, then the routine administration of these antibiotics could reduce the potentiality of broiler chickens as a source of *Campylobacter* infections in humans.

The purpose of this study was to test the susceptibility of chickens to *C. fetus* subsp. *jejuni* by inoculating the bacteria intraperitoneally, intravenously and orally in chickens and to determine the efficacy of two widely used anti-protozoan antibiotics, salinomycin and monensin, to *C. fetus* subsp. *jejuni*.

MATERIALS AND METHODS

Bacterial strains:

Three strains of *C. fetus* subsp. *jejuni* were isolated from humans suffering from gastroenteritis (Shirrow, 1977). The isolates were combined and grown aerobically in brain-heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) at 41°C. Twenty-four hour cultures were stored in the broth at −20°C. Frozen organisms were then thawed and used by the second subculture.

Pure cultures of *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* (Carolina Biological Supply Co., Burlington, N.C.) were used with *C. fetus* subsp. *jejuni* to test the efficacy of antibiotics used routinely in the poultry industry. Twenty-four hour cultures of these bacterial species were stored at 8°C on nutrient agar slants, over-laid with sterile mineral oil until needed.

Experiment I: Chicken Susceptibility Tests.

(i) *Chickens*. Day-old leghorn cockerels obtained from a commercial hatchery were maintained in clean, cardboard, disposable pens and given unmedicated chick starter feed and water *ad libitum*. The room temperature was maintained at 35°C with continuous lighting.

(ii) *Preparation of Inoculum*. Twenty-four hour broth cultures of *C. fetus* subsp. *jejuni* were centrifuged (3000 × g for 10 min.), the supernatent drawn off, and the bacterial cells washed once with sterile saline. The washed bacterial cells were resuspended with sterile saline to obtain the inoculum. A portion of the inoculum was serially diluted in sterile water, inoculated on duplicated 5% sheep blood agar plates (Columbia agar base, BBL Microbiology Systems) and incubated at 42°C in an atmosphere consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen for 48 h to determine colony-forming units (CFU) per ml. The inoculum concentration was approximately 10^7 CFU/ml. Chickens injected intraperitoneally were given 0.2 ml of inoculum. Chickens injected intravenously were given 0.1 ml of inoculum in a wing vein and a 7.5 cm animal feeding needle (Popper and Sons, Inc., New Hyde Park, N.Y.) was used to give 0.5 ml of inoculum orally.

(iii) *Experimental Design*. Seven-day old chicks were selected at random and placed in four groups with six birds/group. Each group was housed in identical cages and given the same feed and water. Group 1 was inoculated intravenously with inoculum. Group 2 was inoculated intraperitoneally and Group 3 was inoculated orally. Group 4 was designated the control group and was not inoculated with *C. fetus* subsp. *jejuni*. Special procedures were maintained to prevent cross contamination. At seven days post-inoculation, the birds were killed by cervical dislocation, the breast smeared with disinfectant and the peritoneal cavity was aseptically opened to expose the viscera. Sterile swabs were taken on the surface of the heart, lungs, intestine and peritoneal lining. Blood and bile samples were also taken. The samples were incubated aerobically in brain-heart infusion broth at 41°C for 48 h. Parallel samples were incubated in 5% sheep blood agar plates at 41°C in 5% oxygen-10% carbon dioxide-85% nitrogen for 48 h. Colonies which morphologically resembled *C. fetus* subsp. *jejuni* were sub-cultured for identification (Leuchtefeld, et al., 1980).

Experiment II: Calculation of minimal inhibitory concentration (MIC) of salinomycin and monensin.

(i) *Antibiotics*. Salinomycin (Pfizer, Inc., Terre Haute, Ind.) and monensin (Elanco Products Co., Indianapolis, Ind.) were obtained from the manufacturers in powdered form. The antibiotics were dissolved in 70% ethyl alcohol and filtered through a sterile 0.45 um membrane filter (Gelman Instrument Co., Ann Arbor, Mich.). One gram of active drug was dissolved in 10 ml of alcohol. One-tenth milliliter of alcohol-drug suspension was mixed with 9.9 ml of brain-heart infusion media to obtain 1000 μg of active drug per 1 ml of media. Serial double dilutions were conducted to obtain a range of drug concentrations and determine the MIC of the drug for each microorganism tested. Alcohol dilutions (without drug) were conducted concurrently to determine if the alcohol alone affected the growth of the bacteria in the media.

(ii) *Standardization of Inoculum*. The turbidity of 24 h broth cultures used for inoculum was adjusted by visually comparing the cultures to that of a 0.5 McFarland turbidity standard before inoculating the serial dilutions with an inoculation loop (National Committee for Clinical Laboratory Standards, 1980). The inoculated serial dilutions of *C. fetus* subsp. *jejuni* were incubated aerobically at 41°C. The other bacterial species (*B. cereus*, *S. aureus* and *E. coli*) were incubated at 37°C.

RESULTS

Chicken Susceptibility Tests.

Chickens were susceptible to *C. fetus* subsp. *jejuni* by all the inoculation methods (Table 1). One chicken inoculated intravenously died on Day 3 postinoculation and three others had active *C. fetus* subsp. *jejuni* infections when they were examined on Day 7 postinoculation. During the postinoculation period, the chickens inoculated intravenously became increasingly listless, pale, inactive and on necropsy all the chickens had enlarged hearts.

Chickens inoculated intraperitoneally had the highest mortality rate with 50% of the birds dying during the postinoculation period. The three remaining chickens has swollen intestines, extensive adhesions and a large focus of infection at the site of inoculations.

Chickens given *C. fetus* subsp. *jejuni* orally had no mortality during the postinoculation period but five of the six chickens had viable *C. fetus* subsp. *jejuni* organisms on the surface of the liver and heart. The intestines, livers and hearts were swollen in all these chickens.

The viscera of all the control birds appeared sterile. None of the birds demonstrated viable *C. fetus* subsp. *jejuni* or any other bacterial species upon autopsy.

Salinomycin and monensin had no bactericidal effect on *C. fetus* subsp. *jejuni* and *E. coli* in the range of concentration tested (Table 2). However, both antibiotics were effective against the Gram positive organisms; *S. aureus* and *B. cereus*. Parallel tests with alcohol controls (without drug) did not inhibit growth of any of the bacterial species at the volumes used in determining minimal inhibitory concentrations of salinomycin and monensin.

DISCUSSION

In every poultry operation, there is spontaneous mortality or the death of birds with no apparent cause. Usually the number of birds involved is relatively small and no pattern to the deaths is apparent so the birds are not examined closely. Most of these deaths occur in the first three weeks of life. When these spontaneous deaths are examined, the majority of birds have swollen intestines and other indications of enteritis. Veterinarians are familiar with the hepatic hemorrhagic lesions associated with *Campylobacter fetus* infections in poultry. However, some of the *Campylobacter* infected birds examined in this study had swollen intestines indicative of enteritis with normal-looking livers. This observation coupled with the fact that *Campylobacter* organisms are fastidious and require special cultural and isolation techniques for identification suggests that some spontaneous mortality in poultry operations may be due to *Campylobacter* infection.

Salinomycin and monensin are examples of polyether antibiotics used by the poultry industry to control coccidial organisms (Reid, 1978). In addition to their anticoccidial action, the polyether antibiotics are extensively used because of minimal tissue deposition, slow development of drug resistant strains of protozoan parasites and low toxicity to the host. Both salinomycin and monensin are added continuously in the feed ration from the day the chicks hatch until five days before the birds are marketed. Although salinomycin is not yet sold in the United States, it is used in many foreign countries at a concentration of 70 parts per million (ppm). Monensin is approved by the Food and Drug Administration as a poultry feed additive at concentrations as high as 121 ppm. It is estimated that 80-85% of

the broilers produced in the United States are fed monensin continuously as a feed additive and the antibiotic is sold worldwide.

Because salinomycin and monensin are antibiotics (produced by *Streptomyces albus* and *S. cinnamonensis* respectively), it is not surprising that they have bactericidal action. This study confirms other reports that polyether antibiotics are effective against many Gram positive microorganisms such as *Staphylococcus aureus* and *Bacillus cereus* (Miyazaki, et al., 1974). However, both antibiotics demonstrated no activity against *Escherichia coli* or *C. fetus* subsp. *jejuni* (Miyazaki, et al., 1974), which are Gram negative bacteria.

The data suggest that chickens are highly susceptible to *C. fetus* subsp. *jejuni* infections and hepatic lesions are not always apparent in active infections. The polyether antibiotics, salinomycin and monensin, used as feed additives, have no bactericidal effect on *C. fetus* subsp. *jejuni*. Therefore, poultry and poultry products have the potential for *C. fetus* subsp. *jejuni* contamination and a source for human campylobacteriosis.

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Table 1. The incidence of *C. fetus* subsp. *jejuni* infections in 7 day old chickens inoculated by various methods.

Group	Method of Administration	Postinoculation Mortality[a]	Positive Visceral[b,c] Culture	% of Survivors Positive for Campylobacter
1	Intravenous	1/6	3/5	60%
2	Intraperitoneal	3/6	3/3	100%
3	Oral	0/6	5/6	83%
4	Control (Uninoculated)	0/6	0/6	0%

[a]Numerator indicates number of birds died postinoculation, denominator indicates the number of birds in the group.
[b]Cultures include visceral swabs, blood and bile cultures.
[c]Numerator indicates number of survivors infected, the denominator indicates the number of survivors in the group.

Table 2. Minimal inhibitory concentration (MIC) of salinomycin and monensin with *C. fetus* subsp. *jejuni* and three other bacterial species cultured in Brain-Heart Infusion broth and incubated aerobically.

	MIC (μg/ml)[b,c]	
Microorganism[a]	*Salinomycin*	*Monensin*
C. fetus subsp. *jejuni*	> 1000.0	> 1000.0
Escherichia coli	> 1000.0	> 1000.0
Staphylococcus aureus	< 7.81	< 7.81
Bacillus cereus	< 0.49	< 0.98

[a]*C. fetus* subsp. *jejuni* was incubated at 41°C while the other bacterial species were incubated at 37°C.
[b]Dilution range was between 1000 and 0.489 μg of drug/ml of culture media.
[c]The MIC's were averages of 3 replicates.

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Food Habits of Bluegills from Heated and Unheated Areas of Coffeen Lake

Dennis Newman
Illinois Natural History Survey
Fox Ridge State Park
Charleston, IL 61920

ABSTRACT

Food habits of bluegills collected from heated and unheated areas of Coffeen Lake were examined. Principal food items of bluegills from the heated station, in order of decreasing index of significance values, were plant material, terrestrial arthropods, gastropods, algae, and chironomids. A comparable list of principal food items from the unheated station included terrestrial arthropods, plant material, chironomids, other aquatic insects, and bryozoans. Diversity values, calculated to provide a qualitative assessment of feeding at heated and unheated locations, demonstrated that the diet of bluegills from the unheated station was consistently more diverse. Seasonal variations in diet reflected the bluegill's opportunistic feeding behavior, as seasonally abundant food resources (such as fish eggs, bryozoans, and terrestrial arthropods) were utilized as major food items. Other investigations have suggested that bluegills from heated locations feed more frequently than those from unheated waters. However, fullness indices calculated for this study indicated that stomach fullness was significantly higher at the heated station for only two of eight collection dates.

INTRODUCTION

A fundamental problem for all animals in an aquatic ecosystem is obtaining sufficient nutrition. For fishes inhabiting thermally enriched environments the problem may be exacerbated by accelerated rates of digestion and maintenance metabolism (Bennett and Gibbons 1972, Graham 1974). These fishes may exhibit several responses to mitigate these problems: migration to areas of cooler temperatures (Tranquilli et al. 1979), transitory forays into unpreferred temperatures to forage (Bennett and Gibbons 1972), or consumption of a larger daily ration. Alternatively, fishes may not be capable of mitigating those metabolic problems, and poor growth may ensue.

This investigation of bluegill feeding dynamics was initiated to increase our understanding of the ecological role of bluegills inhabiting cooling lakes. Specif-

ically, this study was designed to ascertain and compare the diet of bluegills (*Lepomis macrochirus*) from heated and unheated locations of Coffeen Lake.

STUDY AREA

Coffeen Lake was formed in 1963 by impounding McDavid Branch, a tributary of the East Fork of Shoal Creek. The normal operating level (590 MSL) was reached by late 1966, providing 446 hectares of lake surface area. Coffeen Lake is relatively deep compared with other Illinois reservoirs. The Illinois EPA (1978a, 1978b) listed selected parameters for 353 Illinois lakes and reported that Coffeen Lake had a greater mean depth (5.7 m) and maximum depth (17.7 m) than 95% of the lakes studied.

Coffeen Power Station, located in Montgomery County, Illinois, is owned and operated by Central Illinois Public Service Company (CIPS). The two coal-fired generating units produce a gross capacity of 945 MeW. These two units, based on a 70% load rate, have a maximum heat rejection of 4.36 BTU hr^{-1} and establish an average 12°C rise in water temperature across the condensors. The thermal effluent leaves the power station via a 0.9 km-long discharge canal and enters Coffeen Lake by flowing over a hot dam. The cooling loop is 6.6 km from discharge to intake, providing 324 hectares of effective cooling area. The unheated area (Station 4) was partially isolated from the main body of the lake by a railroad causeway which effectively blocked the heated water (Fig. 1). A more complete account of the Coffeen power facilities and lake environment may be found in Tranquilli and Larimore (1981).

MATERIALS AND METHODS

Bluegills were collected from heated (Station 1) and unheated (Station 4) areas (Fig. 1) of Coffeen Lake by means of a 230-volt AC electrofishing unit. All collections were made during daylight hours. The fish were placed immediately on ice and transported to the laboratory. Fish weight and total lengths were measured before the stomachs were removed and stored in 10% formalin. In order to obviate size-specific or age-related variations in the diet (Sarker 1975, Sule et al. 1981) only fish larger than 89 mm were included in the analyses.

Stomach contents were removed, identified, and weighed while moist on a Mettler balance (precision 0.1 mg). The composition of the diet was quantified using percent frequency of occurrence, the gravimetric method, and the index of significance as described by Windell (1971). The index of significance value, which represented the geometric mean of the frequency of occurrence and percent weight (gravimetric) values, allowed each food item to be ranked according to the combined assessment of weight and frequency of occurrence. Diversity indices, as described by Mathur (1977), were calculated for each data set using all identifiable food categories. The fullness index was defined as the wet weight (mg) of stomach contents divided by the weight (g) of the fish from which the stomach was taken.

Three sampling anomalies should be noted. First, collections made during June, July, and August 1979 were accidentally discarded; therefore, new collections were made during those months in 1980. Second, bluegills collected in July and August 1980 were not taken from the area of Station 1, as no fish could be

found there at that time. The collections were taken from areas near Station 1.5 (Fig. 1). Third, during January 1979, the unheated station was ice-covered, and no collection could be made.

RESULTS AND DISCUSSION

Index of significance values showed that food habits of bluegills collected from heated and unheated areas of Coffeen Lake were not greatly dissimilar (Tables 1 and 2). Differences in gastropod and bryozoan utilization were most pronounced, the former being more predominant in stomachs of fish from heated areas and the latter more predominant at unheated sites. Chironomids and other aquatic insects were more prevalent in stomachs from the unheated locations; the differences in use apparently reflected similar differences in abundance of those specific food resources. Another distinction between the two areas was the greater reliance of bluegills from the heated station on plant material and algae. Food items of nearly equal importance at both heated and unheated stations included terrestrial arthropods, microcrustaceans, and fish eggs.

Bluegills from both heated and unheated areas of Coffeen Lake consumed substantial quantities of terrestrial arthropods. However, that fact does not necessarily imply that bluegills were using that resource because autochthonous foods were scarce. Although authochthonous food resources were poor at the heated station, they were abundant at the unheated station (Swadener et al. 1980, Warren and Buckler 1981), and yet terrestrial arthropods were the most important food item of bluegills from unheated waters. Several factors may have contributed to the extensive use of terrestrial arthropods as food by bluegills. First, overhanging forest vegetation dominates the Coffeen Lake shoreline and thus provides an interface between aquatic and terrestrial habitats. Second, the feeding behavior and morphological adaptations of bluegills (Keast and Webb 1966), augment that fish's potential for capturing surface prey. Third, the opportunistic nature by which the bluegill feeds suggests that the most abundant and accessible food items are readily exploited. For instance, bluegills examined in this study from the September collections contained enormous quantities of aphids, almost to the exclusion of all other food items.

Diversity values were calculated to provide a qualitative assessment of feeding at heated and unheated locations (Table 3). Those values demonstrated that the diet of bluegills from the unheated station was consistently more diverse than that of bluegills from the heated area; only during October and November 1978 were diversity values higher at the heated station.

Food habits of bluegills from Lake Sangchris (Sule et al. 1981), a cooling lake in nearby Christian and Sangamon Counties, differed from those of bluegills examined in this study. Bluegills from Lake Sangchris relied more heavily upon microcrustaceans, chironomids, and other aquatic arthropods, whereas bluegills from Coffeen Lake consumed more terrestrial arthropods. In addition, gastropods, bryozoans, and fish eggs, all important components of the diet of Coffeen Lake bluegills, were not found in stomachs of bluegills from Lake Sangchris.

Empty stomachs were uncommon in this study, as only 1.0 and 4.5 percent of the stomachs from the heated and unheated stations, respectively, were empty. To further investigate differential feeding intensity at the two stations, a fullness index determined for each specimen showed that stomachs of bluegills from the

heated station contained significantly (P 0.05) more food by weight during March and June than did the stomachs of bluegills from the unheated areas, but there was no statistical difference between stations for the other six months. Differential feeding intensity of bluegills has been investigated in a cursory manner in other cooling lakes. Sarker (1977) and Sule et al. (1981) found that the percentage of empty stomachs was lower in samples taken from heated locations as opposed to those from unheated locations. That same trend held true for this investigation. Both Sarker (1977) and Sule et al. (1981) suggested that bluegills in heated waters fed more frequently because of higher digestive and metabolic rates. However, mean fullness indices calculated for this study were significantly higher (P 0.05) at the heated station for only two of eight collection dates.

Winter food habits did not differ between heated and unheated areas of Coffeen Lake, and the winter diet of bluegills from the heated station did not differ from published reports of the feeding habits of bluegills from lakes with normal winter water temperatures. Food items of increased importance during winter collections were microcrustaceans and plant matter, whereas utilization of chironomids declined and use of terrestrial arthropods declined precipitously (Fig. 2). Similar winter feeding regimes have been reported for several non-cooling lakes (Moffet and Hunt 1943, Goodson 1965). Probably unique to an Illinois lake was the appearance of fish eggs in the winter diet of bluegills, which were found in stomachs from the heated station in mid-March. At the same time fish eggs were not found in stomachs from the unheated area of the lake. This disparity can be related to the bimodal spawning periodicity of largemouth bass in Coffeen Lake. Largemouth bass from the heated area begin spawning approximately four weeks before bass in the unheated area (Tranquilli and Perry 1981).

During spring, fish eggs became the predominant food item of bluegills in Coffeen Lake. Ninety percent of the specimens from the heated station and 80 percent from the unheated waters contained fish eggs, but bluegills from the unheated station consumed a greater percentage weight and thus exhibited a higher index of significance value. Bryozoans were an important food item in spring at the unheated station but were not found in stomachs from the heated station. Food items which were used extensively during winter, microcrustaceans and plant matter, were less important in spring samples as other food resources became abundant. A distinct disparity existed between heated and unheated collections in the use of the major aquatic insect group, chironomidae larvae. The index of significance value for bluegills from the unheated station was 23.7 and only 4.0 for bluegills from the heated station. However, bluegills from the heated area were at the same time making much greater use of terrestrial arthropods as a food resource.

During the summer months water temperatures at the heated station often exceeded the upper avoidance temperature (33°C) of bluegills (Beitinger and Magnuson 1979) and thus may have altered feeding behavior. During this period, gastropods, which were uncommon during the other seasons, became an important component of the diet at the heated station but were much less important at the unheated station. Principal food items at the unheated station during the summer months were terrestrial arthropods, plant matter, bryozoans, and chironomids.

During autumn, plant matter and terrestrial arthropods were the predominant food items. Gastropods were uncommon, and bryozoans and fish eggs were completely absent from the diet of bluegills. Chironomids, which in other seasons

were more extensively utilized by bluegills from the unheated station, were used to a greater extent by bluegills from the heated area of Coffeen Lake.

Benthic food resources were sparse in the area of the heated effluent (Warren and Buckler 1981), and the major component of the littoral benthic community, oligochaetes, was rarely used as food by bluegills. Thus, bluegills from the heated station shifted to other food resources, some of which have been reported to be of limited value, e.g., algae (Kitchell and Windell 1970). Beitinger and Fitzpatrick (1979) stated that bluegills in natural populations are often food limited, and fish under such constraints can reduce maintenance metabolic costs by seeking lower water temperatures. In Coffeen Lake, intra- and interspecific competition for food and space is probably so severe that many fish were unable to occupy preferred temperature regimes. Multiple factors acting synergistically probably contributed to the stunted condition of bluegills (Perry and Tranquilli 1981) in Coffeen Lake. However, two of the more important factors limiting the growth and affecting the body condition of Coffeen Lake bluegills were the quality of the food resource and accelerated rates of maintenance metabolism.

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Table 1. Composition of the diet of 108 bluegills collected from the heated station of Coffeen Lake, October 1978-August 1980. Food items are listed in order of decreasing importance according to their index of significance value. Mean total length was 126 mm, range 90-154 mm.

Food item	Index of significance	Percent weight	Percent frequency of occurrence
Unidentified organic matter	55.2	35.8	85
Plant material	33.8	16.3	70
Terrestrial Arthropods	32.8	14.0	77
Gastropoda	16.8	13.5	21
Inorganic matter	13.0	5.3	32
Algae	12.3	6.6	23
Chironomidae	11.7	2.0	68
Microcrustaceans	7.6	1.1	52
Other aquatic insects	7.5	1.4	40
Fish eggs	6.3	2.5	16
Fish scales	2.6	0.3	22
Bryozoa	1.3	0.3	6
Astacidae	1.2	0.7	2
Oligochaeta	0.09	0.002	4
		99.8	

Table 2. Composition of the diet of 110 bluegills collected from the unheated station of Coffeen Lake, October 1978-August 1980. Food items are listed in order of decreasing importance according to their index of significance value. Mean total length was 126 mm, range 91-151 mm.

Food item	Index of significance	Percent weight	Percent frequency of occurrence
Unidentified organic matter	54.0	32.8	89
Terrestrial Arthropoda	34.7	18.8	64
Plant material	23.8	14.5	39
Chironomidae	19.6	5.2	74
Other aquatic insects	12.9	3.6	46
Bryozoa	12.1	9.1	16
Fish eggs	9.1	5.5	15
Microcrustaceans	7.1	1.0	51
Algae	6.4	4.5	9
Fish scales	4.7	1.5	15
Gastropoda	3.8	1.8	8
Inorganic matter	2.8	1.6	5
Oligochaeta	——	——	——
Astacidae	——	——	——
		99.9	

Table 3. Monthly diversity (D) of the diet values at heated and unheated stations in Coffeen Lake.

Date	Diversity (D)	
	Heated	Unheated
Oct. 1978	1.99	1.46
Nov. 1978	1.92	0.56
Jan. 1979	1.18	*
Mar. 1979	1.28	1.55
May 1979	1.61	2.46
Sept. 1979	1.78	1.84
June 1980	1.23	2.08
July 1980	2.27	2.77
Aug. 1980	1.95	2.08

*No collection due to ice cover.

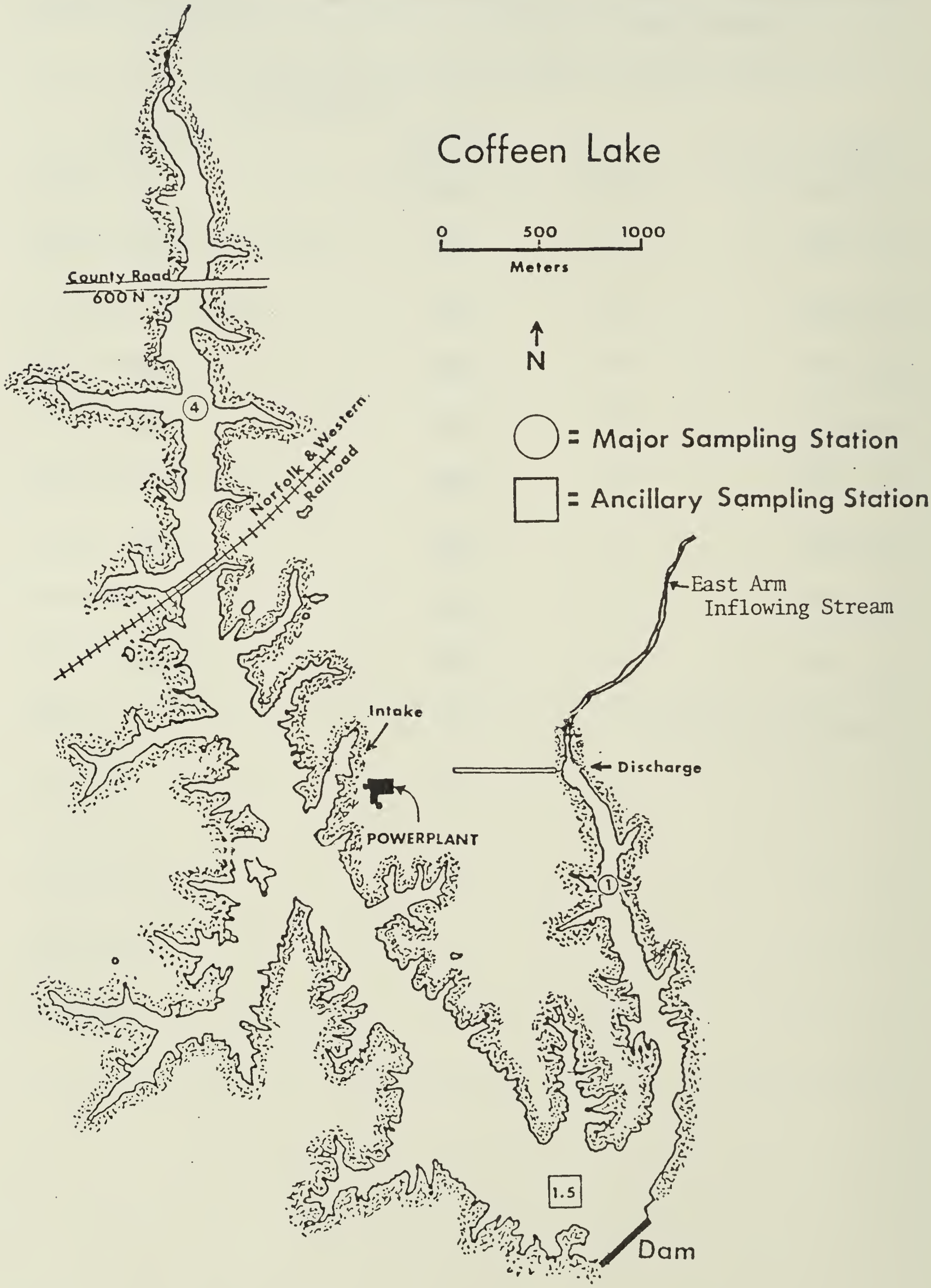

Fig. 1. Sampling stations on Coffeen Lake used during this study (Stations 1, 1.5 and 4).

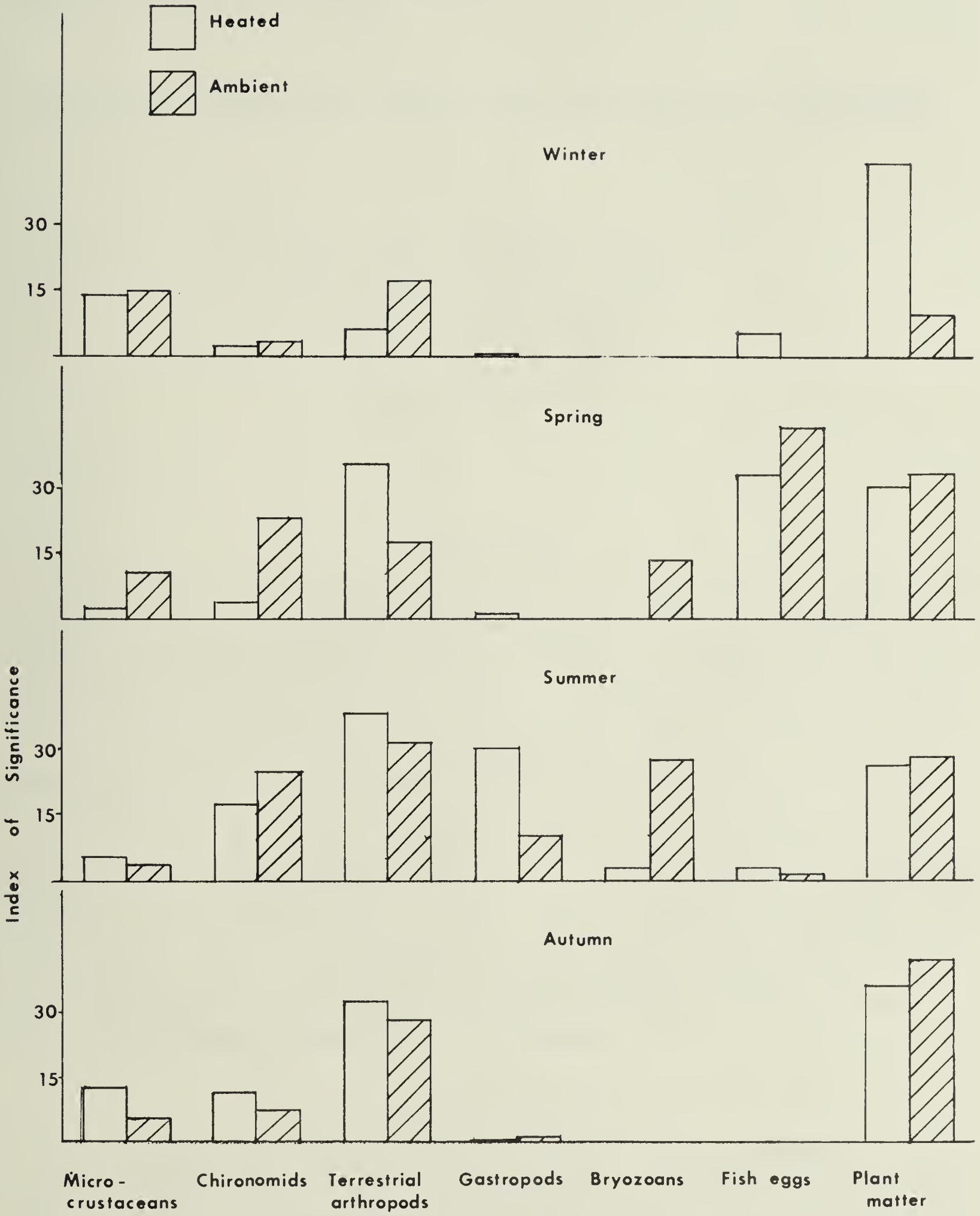

Fig. 2. Seasonal variations in the diet of bluegills from heated and unheated areas of Coffeen Lake.

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Lignin Analyses on Sweet Sorghum Samples

K.D. Carlson, M.E. Carr, R.L. Cunningham,
M.O. Bagby, and D.M. Palmer
Northern Regional Research Center,
Agricultural Research Service,
U.S. Department of Agriculture,[1]
Peoria, Illinois 61604

ABSTRACT

A comparison of lignin contents determined by two methods on two replicated samples receiving one of three pretreatments is reported for freeze-dried sweet sorghum. The pretreatments were: extraction with 95% ethanol/benzene (1:2, v/v); extraction with 80% aqueous ethanol; and no extraction prior to lignin analysis by ultraviolet spectrophotometric (UV) or sulfuric acid methods. Multiway analysis of variance was used to examine results, and highly significant variation among treatments and methods was observed. Differences in lignin content between samples and between methods were greatest when the sweet sorghum was not extracted prior to analysis. These differences were reduced when samples were first extracted with alcohol/benzene. Differences between samples and between methods virtually vanished when an 80% aqueous ethanol extraction preceded lignin analysis. The results illustrate the limitations of these lignin analysis methods when applied to a material like sweet sorghum. A pretreatment consisting of 80% aqueous ethanol extraction appears to provide a suitable substrate for either method of lignin analysis on sweet sorghum.

INTRODUCTION

We recently reported lignin contents of sweet sorghum [*Sorghum bicolor* (Moench), variety Wray] as part of a compositional study on freeze-dried whole stalk and bagasse samples harvested from sewage sludge-amended stripmine soils (Carlson *et al.*, 1983). The method used was that of Bagby *et al.* (1973), in which lignin is solubilized in acetyl bromide and determined by ultraviolet (UV) absorption at 280 nm. In developing this method, these workers showed that the differences in lignin content determined for samples of green kenaf (*Hibiscus cannabinus L.*) both before and after 95% ethanol/benzene (1:2, v/v) extraction was not signifi-

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

cant, but that the precision without the extraction was lower when all data from the study were included (green and post-frost kenaf, and kenaf pulps). The UV method was highly correlated ($r = 0.97$) with a previously developed sulfuric acid gravimetric method (Bagby *et al.*, 1971), but lignin contents determined by the two methods for kenaf pulps were significantly different ($P < 0.05$). Generally, either method has proved satisfactory for determining lignin in nonwood, low-extractive, agricultural-type materials when preceded by the alcohol/benzene extraction step. In this note, comparison of the two methods as applied to sweet sorghum samples shows that results differ significantly depending upon pretreatment of the sweet sorghum samples, and a pretreatment is suggested that gives consistent results by either method of lignin analysis.

MATERIALS AND METHODS

One sample of sweet sorghum (60C, Table 1) was grown in central Illinois as previously described (Carlson, *et al.*, 1983). The second sample (25A, Table 1) was a composite of sweet sorghum grown in five locations in Michigan and received frozen. Fresh (IL) or frozen (MI) stalks cut into short segments were split longitudinally, freeze-dried, and finally ground to pass a 1-mm screen. Each sample contained approximately 50% total sugars (dry basis). Alcohol (95%)/benzene (1:2, v/v) or 80% aqueous ethanol (ethanol/water, 80:20, v/v) extractions were made in a Soxhlet extractor for 18 hr on 10-g samples in duplicate. Solubles removed from sample 60C were 25.1% (alcohol/benzene) and 61.5% (80% aqueous ethanol); from sample 25A, solubles were 51.9% (alcohol/benzene) and 55.7% (80% aqueous ethanol) of the sample dry weight. Lignin analyses were made by the sulfuric acid (Bagby *et al.*, 1971) and UV spectrophotometric methods (Bagby *et al.*, 1973). Data were examined by analysis of variance based on two sorghum samples, two methods, three extraction pretreatments, and two replicates of each combination. The least significant ratio for comparing two means was determined (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Wall and Blessin (1970) tabulated compositional data for different sorghums that had lignin contents ranging from 10% to 17%, the lower values for sweet sorghums and the higher values for grain and grass sorghums. The UV method for lignin determination (Bagby *et al.*, 1973), as applied to our sweet sorghum, similarly gave lignin contents of 12 to 20% for alcohol/benzene extracted, freeze-dried stalks (Carlson *et al.*, 1983). Alcohol/benzene extraction removed an average of 25% of the dry weight of these samples, the same as previously reported for sweet sorghum extractives (Wall and Blessin, 1970).

We became increasingly aware of interfering absorption in UV lignin determinationss on many sorghum samples that are routinely received and screened for sugars and other constituents. We presumed that the problem was associated with the presence of high levels of extractives in many of these samples, and an alternate extraction with 80% aqueous ethanol was devised to remove both sugars and interfering UV absorbers (possibly tannins, etc.). This technique provided substrates with much lower apparent lignin than either unextracted or alcohol/benzene extracted sorghum. We tested the effectiveness of the pretreatments by comparing

the UV lignin analysis against the gravimetric sulfuric acid method for lignin. Ideally, the two methods will agree when interfering UV absorbers have been removed, assuming, of course, that the sulfuric acid method does not destroy lignin and that the extraction solvents neither destroy nor remove lignin from the sample.

The results of our study are shown in Table 1. Mean lignin contents are listed for two samples that received no preparatory treatment or one of two extraction pretreatments prior to lignin analysis. Highly significant variation among treatments was observed. The least significant ratio (LSR) of two means (sample, treatment, or method) is 1.086; two means with different superscripts are significantly different ($P < 0.05$). The overall relative standard deviation among sample replicates was 3.9%, indicating that determinations by either method for a particular sample were highly reproducible. The three- to sixfold range in UV lignin values, depending upon sample and pretreatment, illustrates the unreliability of the method for a high-extractives material like sweet sorghum unless interfering substances are removed with an appropriate solvent. Agreement among the sulfuric acid lignin data, regardless of sample and pretreatment, is substantially better but differences are still significant, suggesting that this method is also sensitive to substances associated with the extractives. Differences in lignin content, by either method, are significant for samples receiving the same pretreatment when the pretreatment is either no extraction or alcohol/benzene extraction. However, when the extractant is 80% aqueous ethanol, apparent lignin contents converge near 5%, and only the UV value for sample 25A is statistically different from the other three values.

This convergence is shown in Figure 1, where apparent lignin contents (%) for the two samples in Table 1 and additional UV lignin data for four other samples are plotted as a function of extractives (%) removed by each extractant. Linear regression analysis on the data gave the equations:

$$\text{Lignin (UV)} = 24.63 - 0.332\ \text{Extractives} \qquad (r = -0.95)$$

$$\text{Lignin (Sulfuric acid)} = 7.29 - 0.0373\ \text{Extractives} \qquad (r = -0.75)$$

UV lignin is highly correlated with extractives. The slope of the sulfuric acid lignin line was not significantly different from zero, but more data are needed to test further the sensitivity of the sulfuric acid method to interference by extractives of sweet sorghum. The sulfuric acid method gives a reasonable estimate of lignin in unextracted sweet sorghum. Convergence of the two methods occurs near 59% extractives with an apparent lignin content near 5.1%.

We found that a second extraction with alcohol/benzene was as effective as a single 80% aqueous ethanol extraction in bringing the apparent UV lignin into line with the sulfuric acid method and the 80% aqueous ethanol value. For example, on the same sorghum sample, UV lignin of 10.4% and 35.9% extractives were determined in the single alcohol/benzene extraction; after the second alcohol/benzene extraction the values were 5.4% lignin and 56% extractives, compared with 5.5% UV lignin and 59.2% extractives in the single 80% aqueous ethanol extraction.

It is conceivable that 80% ethanol extraction or treatment with sulfuric acid removes or destroys lignin, which would result in low lignin values by either method. It is also possible that agreement between methods is fortuitous when the extractant is 80% aqueous ethanol. Results given in the previous paragraph argue against either of these possibilities. From our results it is apparent that 80%

aqueous ethanol extraction of sweet sorghum provides a suitable substrate for obtaining comparable apparent lignin contents either by the faster UV method or by the more consistent sulfuric acid method. Exhaustive alcohol/benzene extraction is also effective in preparing sweet sorghum samples for UV lignin analysis.

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Table 1. Apparent lignin contents of freeze-dried sweet sorghum stalks as determined by two methods on samples receiving different solvent extractions prior to analysis.

	Lignin, % (Dry Basis)*			
	UV Method#		Sulfuric Acid Method+	
Extractant‡	Sample 60C	Sample 25A	Sample 60C	Sample 25A
None	30.36[a]	19.80[b]	5.78[f]	8.52[d]
95% Ethanol/benzene (1:2, v/v)	16.68[c]	7.32[e]	6.92[e]	5.12[g]
80% Aqueous ethanol (80:20, v/v)	5.27[g]	6.16[f]	5.09[g]	5.08[g]

*Means of duplicate analyses; means with different superscripts are significantly different ($P < 0.05$). Least significant ration (LSR) of means = 1.086. Relative standard deviation (RSD) = 3.9%.
#Method of Bagby *et al.* (1973), by ultraviolet (UV) absorption at 280 nm.
+Method of Bagby *et al.* (1971).
‡Soxhlet extractions (18 hr), prior to analysis.

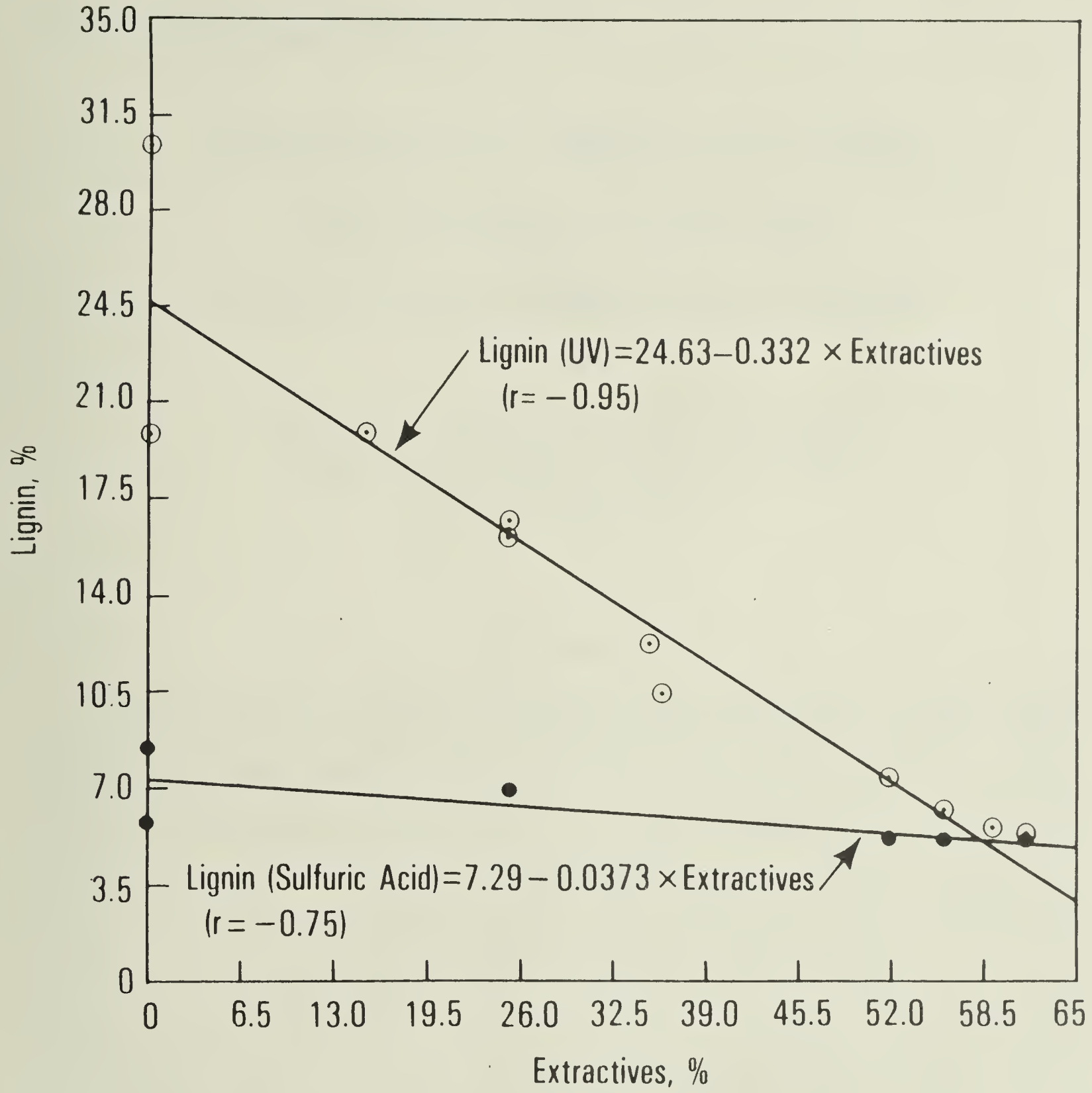

Fig. 1. Apparent lignin contents of sweet sorghum samples as a function of extractives (% of sample dry weight solubilized during alcohol/benzene or 80% aqueous ethanol extraction).

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Abundance of Whip-poor-wills, *Caprimulgus vociferous*, In the Sand Ridge State Forest

Richard G. Bjorklund and Eric R. Bjorklund
Department of Biology, Bradley University
Peoria, Illinois 61625

ABSTRACT

A standardized precedure for censusing caprimulgids (Aves: Caprimulgidae) was established in the Sand Ridge Forest in 1981 and repeated in 1983. Calling-counts were made from seven listening stations along a prescribed five kilometer route through representative forest cover types. Three species of caprimulgids summer on the Forest, but only Whip-poor-wills, *Caprimulgus vociferous*, were numerous enough to determine (1) conditions favoring reliable calling-counts, and (2) abundance in various forest cover types. Calling occurs from April through August, but June is the best month for censusing. The number of calls detected was maximal on moonlit nights when winds were calm or very light, not exceeding Beaufort wind code 3 (8-12 mph). Moonlight, and perhaps high humidity, enhanced vigor of calling. In June, number and vigor of calls was unaffected by temperatures within the range of 8-27°C; however, rainfall markedly reduced calling. Counts completed 30 minutes before sunrise yielded virtually the same results as counts initiated 30 minutes after sunset. For both years, calling-counts were greatest at a station surrounded by mixed pine and oak cover interspersed with wildlife food and cover plots and a campground; oak stands were intermediate, and a station surrounded by pine plantations lowest, in calls recorded. Based upon the highest June count, approximately 220 Whip-poor-will territories were established on the forest in 1981 and 188 territories in 1983. Estimates based upon the average of six June counts made under favorable conditions were 202 territories in 1981 and 158 in 1983.

INTRODUCTION

The Sand Ridge Forest, located in Mason County, is a 3,000 hectare peninsular island of sand scrub hardwoods, conifer plantations, and mixed stands surrounded almost completely by agricultural land. Only a narrow forested isthmus at its

northwest corner connects the Forest with the Illinois River Valley. Members of three species of caprimulgids (Aves: Caprimulgidae), all more often heard than seen, are summer residents of the Forest. They are: Whip-poor-will, *Caprimulgus vociferous*, Chuck-will's-widow, *Caprimulgus carolinensis*, and Common Nighthawk, *Chordeiles minor*. In 1981 the Illinois Department of Conservation funded a survey of the breeding birds of the Forest. As part of that survey, we established a transect for spot censusing caprimulgids from calling-counts in order to determine the abundance and habitat preferences of the three species present. The calling-count procedure initiated in 1981 was repeated in 1983. However, only Whip-poor-wills were numerous enough to determine (1) conditions favorable to reliable counts, and (2) abundance in various forest cover types. Information on behavior and nesting, obtained for all three species, is being reported separately (Bjorklund and Bjorklund, 1984).

METHODS

Standard methods prescribed by the Department of Conservation for censusing breeding birds were necessarily modified for caprimulgids, inasmuch as they are predominately crepuscular and nocturnal, although nighthawks are sometimes active during the day and are a common sight in urban areas with flat roofed buildings. Counts were made by listening for species specific calls at seven stations located at 0.8 km intervals along an unpaved sand road (Sand Ridge Road) which runs east-west through the Forest. The most easterly station was established near the Pine Campground; the most westerly, at the west edge of the Forest. The time spent listening at each station was standardized at 3 min. The counts were started from alternate ends of the route in successive count periods, each of which began with 30 min. of sundown or early enough to conclude 30 min. before sunrise. Until July 1983, counts were paired to make morning-evening comparisons possible. The station intervals and the listening time chosen were fortuitous for, after filing our 1981 field report with the Department of Conservation, Cooper (1981) published his study of the abundance of caprimulgids in Georgia in which he used an identical procedure, except that he began all of his counts at sundown.

Pertinent environmental information — temperature, wind and sky conditions, phase and timing of rising and setting of the moon, humidity, and precipitation were recorded for each census along with the number of calls and field notes on the vigor of calling at each station.

In aggregate, the cover types associated with the seven stations, closely approximate the principal cover types for the forest, which are: 54% deciduous sand forest, predominantly black oak, *Quercus velutina*, and blackjack oak, *Quercus marilandica*; 35% pine plantations, predominantly jack pine, *Pinus banksiana*, red pine, *Pinus resinosa*, white pine, *Pinus strobus*, pitch pine, *Pinus rigida*, and Virginia pine, *Pinus virginiana*; and 10% brush, crop and forage, and developed areas (campgrounds, fish hatchery). The remainder consists of sand prairie and ponds. All of the types are crossed or encircled by firebreaks, which along with several sand roads, create considerable weedy and brushy edge.

RESULTS

Calling occurs from April to August; but June is the best month for censusing, as territories are well established. Results for counts made under various condi-

tions are summarized in Table 1. Unless otherwise noted, assume that winds were calm (Beaufort code 0) or were very slight (Beaufort 1). Only temperature extremes are noted in the table.

During June in both 1981 and 1983, maximum counts were obtained when winds were calm and the moon one quarter to full, although a maximum count was also obtained on the morning of 10 June 1981 after the moon had set, and a high count was recorded on 3 June 1981 when the moon was new. A good demonstration of the effect of moonlight occurred on 15 June, 1983 when a heavy cloud bank completely obscured the moon during counts at the last two stations reducing them from the total of six or seven obtained under moonlit conditions to a total of three calls of reduced vigor. Temperature in June, within the wide range noted in the table, had no effect on the number of calling Whip-poor-wills. However, the vigor of calling was noticeably enhanced on warm humid nights, though moonlight was also a factor in each such instance. While high humidity did not lessen calling of Whip-poor-wills, light rainfall did.

In Sand Ridge Forest, winds of Beaufort 3 or greater generate leaf rustle in deciduous stands and equivalent sounds in pine plantations, sufficient to reduce detectability of calling birds. The moderate velocities (12-15 mph) we experienced on 14 June 1981, may not reduce calling; however, data obtained during winds of even moderate velocity are unreliable because of the detectability problem.

The effect of calendar date is indicated in the reduced calling totals for July, despite otherwise favorable conditions.

Table 2, which displays results of six counts made under favorable conditions for each of the years 1981 and 1983, shows that there is no discernable difference between morning and evening counts made when conditions are favorable. It also shows that mixed cover, especially when food and cover plots or exceptional amounts of forest edge are present (stations 1, 2, 5, and 7), produced consistently higher totals of calling in both years. Deciduous stands dominated by oak trees (stations 3 and 4) ranked second, while a station (6) surrounded by pine plantations produced the lowest calling-count in both years. Lower total calling-counts obtained at every station in 1983 compared with 1981, strongly suggest a lower breeding population of Whip-poor-wills in 1983.

DISCUSSION

Although we found near zero wind velocity most important for reliable censusing of Whip-poor-wills and other caprimulgids from calling-counts, moonlight does appear to have a positive effect on the number of individuals calling and the vigor of calling in Whip-poor-wills. Cadbury (1981) discounted moonlight as an enhancing factor in calling of the Nightjar, *Caprimulgus europaeus*, in the English countryside; however, Cooper (1981) documented the positive effect of moonlight particularly well by monitoring calls of Whip-poor-wills and Chuck-will's-widows before, during, and following a lunar eclipse. Without reporting the wind velocities prevailing during his observations, Cooper discounts wind as a factor affecting the results of calling-counts of caprimulgids. In contrast, we are in agreement with Cadbury (1981) who reported that winds above Beaufort 3 (8-12 mph) adversely affect detectability of calling in nightjars. We are also in agreement with both Cooper and Cadbury regarding the negative effects of rainfall on calling.

With regard to habitat perference, Cooper (1981) found that the Whip-poor-

will, which is extending its range into Georgia, showed a preference for wooded habitat while the Chuck-will's-widow showed an affinity for open areas at the edge of woods. Thus the two species are unlikely to compete for breeding territories, even when present in the same general cover type. This is of interest to us because the Chuck-will's-widow, a rare migrant and summer resident in central Illinois (Bohlen, 1978), was not detected on the Forest until 1975 (Bjorklund, 1981). Over-migrants of this species are establishing a small breeding population in a somewhat open area of the Forest which resembles descriptions of its preferred habitat (Bent, 1940). However, in the open areas at Sand Ridge Forest, the Chuck-will's-widow may be brought into conflict with the Common Nighthawk (Bjorklund and Bjorklund, 1984).

The number of Whip-poor-will territories on the Forest may be estimated using data from Table 2 and an assumption for spot censusing other species of birds by the Illinois Department of Conservation procedure. We assume that under favorable conditions Whip-poor-wills, like most other avian species, can be detected from an area of approximately 64 hectares (160 acres) surrounding each station; thus, we estimate that in 1981 there were 202 Whip-poor-will territories on the 3,000 hectare forest, while in 1983 the number of territories was 158. If we use only the highest June count for each of the two years, the estimated number of territories is 220 in 1981 and 188 in 1983. The decline in calling was apparent at all sampling stations. Its cause is unknown to us.

The average number of calling Whip-poor-wills per station in June was 4.3 in 1981 and 3.3 in 1983. In forest habitat in Georgia, the average number of calling Whip-poor-wills per station in June 1981 was approximately 3.2, but if the number of calling Chuck-will's-widows is included, the average in about 4.2 for caprimulgids.

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Table 1. Number of calling Whip-poor-wills under various conditions, Sand Ridge Forest, 1981 and 1983.

Time	1981	Noteworthy Conditions	Number Calls	1983	Noteworthy Conditions	Number Calls
AM	3 June	ground fog	28	1 June	moon last quarter; 8°C	25
PM	3 June	new moon	30	31 May	prior to moonrise; 10°C	17
AM	10 June	moon set	33	——	no equivalent count; wind interference	——
PM	10 June	thunderstorm approaching	26	——	no equivalent count; wind interference	——
AM	15 June	moon set; wind code 2	24	14 June	moon set; wind code 2	22
PM	14 June	wind code 3-4; near full moon	21	15 June	clouds obscured moon last two stations	17
AM	17 June	moon setting; 11°C	31	19 June	light rain	2
PM	16 June	full moon	33	18 June	light rain	6
AM	——	——	——	25 June	full moon setting; humid	23
PM	——	——	——	22 June	near full moon rising; very humid; 27°C	26
PM	——	——	——	23 June	full moon overhead; very humid; 25°C	28
PM	——	——	——	15 July	first quarter moon; 27°C	17
PM	——	——	——	22 July	full moon; humid; 32°C	15

Table 2. Number of Whip-poor-wills heard calling under favorable conditions in various cover types; three morning and three evening counts each station, June 1981 and 1983.

		June 1981			June 1983			
Station	Type Cover	Total Calls	AM	PM	Total Calls	AM	PM	Combined
1	pine, oak, main campground, wildlife food and cover	33	19	14	26	12	14	59
2	pine, oak, wildlife food and cover	24	13	11	20	12	8	44
3	oak, small campground	24	11	13	18	7	11	42
4	oak	23	10	13	21	11	10	44
5	oak, pine	26	14	12	20	8	12	46
6	predominantly pine plantations	21	10	11	16	9	7	37
7	pine, oak much edge (west margin forest)	30	15	15	20	11	9	50
Totals		181	92	89	141	70	71	322

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A Digest of Water Quality of Three Central Illinois Impoundments

Shun Dar Lin and Ralph L. Evans
Water Quality Section
Illinois State Water Survey
Box 697, Peoria, IL 61652

ABSTRACT

Samples from Lakes Canton, Decatur, and Eureka were collected weekly for over 2 years and were analyzed for 22 parameters. These parameters are transparency, water temperature, dissolved oxygen (DO), turbidity, pH, alkalinity, hardness, ammonia and nitrate nitrogen, dissolved and total phosphorus, iron, manganese, sulfate, chlorine demand, chemical oxygen demand (COD), total organic carbon (TOC), silica, threshold odor number (TON), algae, standard plate count (SPC), and actinomycetes. Presented are summaries of the comparisons of water quality characteristics, 1) with different depths, 2) with different lakes, and 3) with Illinois Pollution Control Board's standards.

INTRODUCTION

The three man-made impoundments serve principally as sources of public water supplies and as public recreation areas. Lake Decatur has a large surface area (1170 ha) and a maximum depth of 5 m. Lake Eureka is a small body (15 ha) of water with a maximum depth of 7 m. Lake Canton has a mid-size surface area (88 ha) and a maximum depth of about 11 m.

Over a 2-year period, mid-September 1976 to mid-October 1978, water samples from these three impoundments were collected weekly at the deepest locations. Sample collections were made at the surface, mid-depth, and near bottom at each location and were analyzed for 22 physical, chemical, and microbiological constituents.

This paper is a summary of the findings of the study. Taste and odor portions have been reported elsewhere (Lin and Evans, 1981a, 1981b). The observed data are available from computer storage at Illinois State Water Survey.

RESULTS AND DISCUSSION

Table 1 shows average values and geometric means of the observed data depending on distribution. The transparency in Lake Canton was generally greater than that in Lakes Eureka and Decatur. Low transparency occurred in mid-spring and late summer.

The water temperature exhibited seasonal changes throughout the water column. The water of L. Canton and L. Eureka was thermally stratified for about six months every year from mid-April to mid-October. The temperature stratification barrier for L. Canton and L. Eureka occurred at 12 to 14 feet (3.7 to 4.3 m) and 4 to 10 feet (1.2 to 3.0 m), respectively.

During the winter and between the spring and fall, lake turnovers, stratification in terms of dissolved oxygen occurred in all three lakes. The lower strata of water in L. Canton and L. Eureka were void of dissolved oxygen during the summer stagnation period. There was never a void of dissolved oxygen in Lake Decatur. During the summer stagnation period (June to mid-October 1978) for about 70 percent of the time the anaerobic zone in L. Canton and L. Eureka extended into the overlying water 14 to 20 feet (4.3 to 6.1 m) and 6 to 8 feet (1.8 to 2.4 m), respectively, from the lake bottom. The thermal stratification of the lakes was not a barrier to the anerobic zone penetrating the overlying waters.

In general, except during the summer stagnation periods, hardness, COD, and TOC in all lakes did not change in water column. In L. Decatur concentrations of pH, alkalinity, nitrate, sulfate, chlorine demand, and silica did not change on the vertical.

At the surface and mid-depth of L. Canton and L. Eureka, the turbidity, alkalinity, nitrate nitrogen, and sulfate concentrations were not significantly different. There was no difference in dissolved and total phosphorus concentration at the surface and mid-depth in each of the three lakes. For the upper layes (surface through mid-depth) the stable constituents were: ammonia-nitrogen in Lake Decatur, manganese in L. Decatur and L. Eureka, and chlorine demand in Lake Canton. The concentrations of these parameters at the deep waters were significantly greater than that at the upper half stratum in each lake.

Concentrations increasing with water depth were as follows: iron, standard plate count, and actinomycete densities in all three lakes; ammonia nitrogen, manganese, and silica in Lake Canton; ammonia nitrogen, chlorine demand, and silica in Lake Eureka; and turbidity in Lake Decatur. In contrast, algal densities in each lake decreased significantly with water depth.

In Lake Decatur, there were no trends for temporal variations of all water quality characteristics except algae, alkalinity, and turbidity. Turbidity in Lakes Decatur and Eureka were higher in the summer. However, no seasonal pattern of turbidity was observed in the deeper portion of Lake Canton. Alkalinity values in Lake Decatur were higher in the winter. Hardness, pH, COD, TOC, silica, and actinomycete in Lakes Canton and Eureka exhibited no temporal variations. The water in Lake Canton can be classified as moderate-hard. Lakes Decatur and Eureka are hard waters.

During the summer stagnation period in L. Canton and L. Eureka the hypolimnion became anaerobic. The phenomena at the lower strata of water in L. Canton and L. Eureka were the increases of turbidity, alkalinity, hardness, ammonia-nitrogen, dissolved and total phosphorus, iron, manganese, chlorine demand, silica, and SPC, and the decreases of pH, nitrate-nitrogen, and sulfate. These phenomena were due to bacterial fermentation and decomposition. At the surface waters, in contrast, alkalinity and silica decreased; and pH and algae increased due to active algal growths. *Aphanizomenon flos-aquae* and *Ceratium hirundinella* were the predominant algae in Lakes Canton and Eureka; respectively. *A. flos-aquae* dominated in Lake Decatur during the summer.

Vertical migration of silica in Lakes Canton and Eureka was much less than that of phosphorus and iron. The vertical limit of ammonia-nitrogen concentrations was consistent with the limit of the anaerobic zones. The releases of phosphorus and iron at the bottom of Lake Decatur were sporadic and unpredictable. Turbidity contents at the deep waters in Lake Decatur were higher in the summer due to sedimentation.

The magnitude of taste and odor in the three lakes was found to be a seasonal function. High TONs generally occurred during the period from May through October, the period of anoxic conditions in bottom waters of impoundments. Regardless of the magnitude of TONs for the raw waters, the TONs for the three water treatment plants finished waters ranged from 3 to 10 about 95 percent of the time.

Water temperature and sulfate concentrations in the three lakes were well within the IPCB limits. Ten surface water samples collected from Lakes Canton and Eureka exceeded the stipulated pH limit of 9.0. Nitrate nitrogen values in Lake Decatur exceeded the IPCB standard of 10 mg/1, only during mid-May to mid-June 1977 (about 3 percent of the total samples). Some samples taken from the mid-depth and deep stations of L. Canton and L. Eureka did not meet the ammonia nitrogen limits.

The concentrations of total phosphorus in the upper strata of L. Canton and L. Eureka exceeded the limit of 0.05 mg/1 for about 50 and 75 percent of the time, respectively. For the remaining stations, total phosphorus concentrations exceeded the limit more than 90 percent of the time. The iron concentrations of the upper stratum of Lake Canton violated the limit of 0.3 mg/1 about one-third of the time. At all other locations iron concentrations violated the limits 54 to 90 percent of the time. Manganese contents in the three lakes met the limit of 1.0 mg/1 most of the time except at the deep waters in L. Canton and L. Eureka. About one-third of samples from each lake exceeded 500 counts/ml of SPC limit.

Most of the water quality parameters exhibited no seasonal or yearly variation. There was no correlation between parameters except for TON vs. nitrate, sulfate, manganese, and chlorine demand. Parameter concentrations are the results of a very complex inter-reaction. Much of these inter-reactions are still unknown. Thus, modeling a complicated natural system should be discouraged until the nature and rates of interactions are better defined.

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Table 1. Averages or geometric means of observed data.

Parameter	Lake Canton			Lake Decatur			Lake Eureka		
	Surface	Mid-depth	Deep	Surface	Mid-depth	Deep	Surface	Mid-depth	Deep
Transparency, cm		113			80			83	
Temperature, °C	15.2	12.0	9.0	13.7	13.4	13.1	16.1	13.3	11.7
Dissolved oxygen, mg/l	9.5	6.0	2.9	9.9	9.1	8.0	9.6	6.4	4.1
Turbidity, NTU	2.8	2.6	7.3	4.5	6.5	9.8	2.3	3.5	7.4
pH, median	8.3	8.1	7.8	8.3	8.3	8.2	8.4	8.2	8.0
Alkalinity, mg/l as $CaCO_3$	135	145	190	195	196	197	233	242	263
Hardness, mg/l as $CaCO_3$	220	232	250	298	301	300	278	296	300
Nitrate-N, mg/l	0.79	0.83	0.61	3.58	3.58	3.58	2.71	2.78	2.73
Ammonia-N, mg/l	0.16	0.35	1.30	0.05	0.05	0.06	0.11	0.15	0.52
Phosphorus, mg/l									
Dissolved*	0.018	0.020	0.060	0.040	0.044	0.052	0.018	0.016	0.023
Total*	0.050	0.055	0.150	0.11	0.11	0.13	0.080	0.088	0.141
Iron*, mg/l	0.17	0.23	0.72	0.42	0.51	0.99	0.31	0.38	0.99
Manganese, mg/l	0.08	0.16	0.85	0.08	0.08	0.11	0.09	0.08	0.34
Sulfate, mg/l	52.0	51.0	44.6				40.3	40.0	34.6
Sulfate*, mg/l				48.5	48.0	47.0			
Chlorine demand, mg/l	5.2	6.0	9.6						
Cl_2 demand, mg/l				2.2	2.0	2.1	3.6	4.3	7.8
COD, mg/l	10.4			6.8			16.1		
COD*, mg/l		12.0	13.5		11.5	12.8		19.2	21.0
TOC*, mg/l	8.0	7.4	8.2	6.8	6.6	7.0	10.9	9.1	11.2
Silica, mg/l				5.7	5.0	5.4	5.3	6.3	7.8
Silica*, mg/l	2.7	3.4	6.1						
TON*	43	49	130	45	52	67	59	68	100
Algae*, organisms/ml	340	200	96	230	140	63	230	120	72
SPC*, organisms/ml	260	420	630	400	470	560	310	410	510
Actinomycetes*, organisms/ml	44	81	210	100	150	210	59	79	140

*Geometric mean

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Winter Foods of Mink In Southern Illinois

John E. Casson[1]
and
W.D. Klimstra
Cooperative Wildlife Research Laboratory
Southern Illinois University
Carbondale, Illinois 62901

ABSTRACT

A total of 319 mink carcasses was collected from 7 counties in southwestern Illinois during the 1974-75 and 1975-76 trapping seasons. Food items were recorded in 280 digestive tracks with mammals (71%) the most frequently recorded. Other foods of importance included fish (23%), amphibians (21%), and crayfish (14%); no vegetative matter was observed. The occurrence of mammals increased from November to January while fishes decreased over the same period. Amphibians and crayfish showed noteworthy increases November to December and slight decreases December to January. Females consumed more small mammals (74%) than males (46%), while larger mammals were more prominent in males (22%) than in females (10%). Fish appeared more often in males (27%) than in females (11%) whereas amphibians and crayfishes were similar in occurrence.

INTRODUCTION

Mink (*Mustela vison*) food habit investigations in California (Grinnell et al. 1937), New York (Hamilton 1936, 1940, 1959), Missouri (Korschgen 1958), Michigan (Sealander 1943), North Carolina (Wilson 1954), and northcentral United States (Errington 1943, 1954) demonstrate a highly variable carnivorous diet. A significant dietary adaptability for various ecological situtations has been demonstrated also for the feral mink in Europe (Akande 1972, Day and Linn 1972, Erlinge 1972, Gerell 1967, 1968).

Because there has not been study of the mink in Illinois, and previous investigations in the midwest were conducted 15 or more years ago, it seemed appropriate to evaluate and report this mammal's diet when the opportunity occurred. This research effort included determination of food habits in southern Illinois late November through early January 1974-75 and 1975-76, and assessment of variation in diet according to sex.

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[1]Present address: U.S. Forest Service Quinault Ranger Station
P.O. Box 56, Quinault, Washington 98575

STUDY AREA

Carcasses of minks were available from furbuyers and trappers representing a 7-county area in southwestern Illinois (Figure 1). St. Clair, Randolph, Washington, and Perry counties are representative of the Till Plains Section of the Central Lowland physiographic division (Leighton et al. 1948) which reflects low relief with streams of low gradation. These river bottomlands, once heavily forested, are now largely in agriculture with only 15-20 percent of the area in forest. Jackson, Union, and Johnson counties of the Shawnee Hills Section of the Interior Low Plateau physiographic division reflect moderate relief with many bluffs and rock outcrops. Streams are mostly fast and clear with rocky substrata (Smith 1961). Habitats exhibit the influence of southern species of flora and fauna (Vestal 1931, Smith 1961). About 30 percent is now forested and agriculture reflects livestock grazing and cash grain farming.

METHODS

The digestive tracts of mink carcasses representing the 1974-75 and 1975-76 furbearer trapping seasons were collected from furbuyers and trappers. The analyses of these tracts to determine food habits generally followed procedures described by Korschgen (1969). Identification of food items emphasized bones, teeth, hair, scales, and feathers and was aided by Mayer (1952), Lagler (1947), Smith (1961), and reference collections of the Cooperative Wildlife Research Laboratory.

Stomach and large intestine contents were analyzed separately for carcasses collected in 1974-75. However, the procedure was terminated when separate meals could not be distinguished; subsequently, each digestive tract was evaluated as a single sample. Analyses were based on percent frequency of occurrence as volume measurements were considered unreliable (Scott 1941 and Mohr 1935). It was Mohr's (1935) opinion that predation should be measured by number of occurrences, not bulk.

RESULTS AND DISCUSSION

Of 319 digestive tracts examined, 162 and 157 represented 1974-75 and 1975-76, respectively. Food items occurred in 280 tracts. Vegetative materials (leaves, bits of twigs, grass, or wood) commonly encountered were not considered food items as these were believed ingested accidently while feeding or struggling in traps (Errington 1943, Sealander 1943, Hamilton 1959). Most samples contained evidence of mink which probably represented self-ingestion when in the trap or when preening; also, contamination probably occurred during pelt removal. Those samples containing only mink evidence were considered empty.

Mammals represented the most frequently occurring food item, appearing in 71% of the samples (Table 1). Other prominent foods were fishes (23% of samples), amphibians (21%), and crayfish (14%). Similar results were recorded by Korschgen (1958) in Missouri and Hamilton (1936, 1940) in New York. Other studies (Grinnell et al. 1937, Wilson 1954, Hamilton 1959, Gerell 1967, 1968, Akande 1972) ranked fishes the most prevalent food item with mammals, birds, crayfishes, and amphibians of varying importance.

Small mammals occurred in 48% of samples and represented over two-thirds of

the frequency of occurrence of this group. Deer mice, rabbits, prairie vole, and house mouse, taken most frequently, are abundant in southern Illinois (Hoffmeister and Mohr 1957). The pine vole and the muskrat appeared in 5% of the samples and occurrence of the latter agrees with findings of Hamilton (1959), Korschgen (1958), and Wilson (1954). Errington (1943) recorded heavy predation when muskrats were made vulnerable by habitat deterioration. Evidence that mink may devour occupants of an entire nest was occasionally encountered; 1 stomach contained 2 adult and about 7 young deer mice.

Fish, the second most important food, were represented primarily by sunfishes and minnows. Most occurrences of the latter included many specimens 2-4 cm in length; suckers and shad were documented frequently. In late autumn and early winter throughout southern Illinois, shad populations commonly experience a winter die-off (pers. commun., R.C. Heidinger, Cooperative Fisheries Research Laboratory, Southern Illinois University, Carbondale.) Such a die-off probably occurred during this study as these fish prefer limnetic waters (Lagler et al. 1962) that make them normally unavailable to mink. Some fish ingestions may have been that discarded by fishermen as sunfish and suckers are commonly left along shorelines.

Many occurrences of amphibians were bone fragments and detailed identification was not often possible. *Rana* and *Acris*, the only genera positively identified, were the major contributors. Egg masses were occasionally encountered along with *Rana*, and on a few occasions a stomach was completely filled with eggs. Day and Linn (1972) documented mink consumption of frog spawn in England. Hamilton (1959) reported amphibians important in the winter diet of mink and concluded mink sought torpid individuals from pond bottoms and stream beds.

Crayfish contributed substantially to the mink diet; a finding similar to that of Hamilton (1936, 1959), Sealander (1943), Wilson (1954), Korschgen (1958), and Gerell (1968). Korschgen (1958) believed mink predation on crayfish was enhanced by increased vulnerability at times of low water levels.

Birds occurred only occasionally with most being small passerines; one sample containing a sparrow was believed carrion as maggots were present. Korschgen (1958), Hamilton (1940, 1959), and Sealander (1943) considered birds of minor importance while Wilson (1954), Gerell (1968), Akande (1972), and Day and Linn (1972) recorded birds a major contribution to the mink diet.

Insects, which occurred as occasional food items, were represented largely as single individuals; they were probably of little importance during the fall-winter period. Some insects were believed incidental ingestions; 1 occurrence of fly larvae was associated with carrion, and 2 others were single soldier flies (*Stratiomyiidae*). Grasshoppers and crickets occurred most frequently; some forms were considered secondary ingestions. Large spiders which occurred in 4 tracts suggested they were occasional food items. Snakes were recorded in 2 digestive tracts.

MONTHLY DIETARY TRENDS

Comparison of diets for November, December, and January did not reveal trends between late autumn and winter; however, changes in occurrence of some important food items were noted (Figure 2). Mammals increased from 69% in November to 79% in January, largely due to rabbit which changed in occurrence

from 11% in November to 18% in January. Hunting pressure on rabbit may have increased in late December and January and a greater number of dead and crippled rabbits may have become available. The fact that habitat use of mink and cottontail tends to overlap in bottomlands during winter (Scott and Klimstra 1955) supports this theory.

The occurrence of fish decreased proportional to the increase of mammals, an observation coinciding with that of Gerell (1967, 1968) and Akande (1972). The former hypothesized fish experienced increased vulnerability when there was metabolic slowdown during cold water temperatures resulting in decreased activity.

Amphibians and crayfish increased substantially in December but declined in January; change in the latter was significant ($X^2 = 4.15, P < .05$). In contrast Gerell (1968) and Day and Linn (1972) reported the consumption of crayfish decreased during winter months. Gerell (1968) believed a decrease in crayfish activity was responsible. Seemingly, a decrease in their activity would increase vulnerability as crayfish burrow in the benthic zone of ponds and streams rendering themselves easy prey. Because amphibians lie dormant in the benthic zone during winter (Hamilton 1959), they may also become more vulnerable to mink predation. Such findings support Wilson (1954) who recorded a substantial occurrence of amphibians in winter. In contrast, Hamilton (1940) observed large numbers of frogs in summer but recorded few in the diet of mink.

COMPARISON OF MALES AND FEMALES

A comparison of diets by sex (Table 2) showed that females consumed more small mammals (74%) than males (46%) ($X^2 = 7.57, P < .01$); occurrence of larger mammals (muskrat and rabbit) was the reverse (22% for males and 10% for females) ($X^2 = 3.56, P < .10$). These results agree with Sealander (1943) although he did not observe differences in the occurrence of rabbit. Hamilton (1959), who found muskrat and rabbit noticeably more frequent in males, contributed this to the larger size and greater mobility of male mink. Sealander (1943) proposed that females take smaller prey because they are easier to capture while larger males can handle larger prey.

Overall occurrence of fish was greater in diets of males ($X^2 = 4.93, P < .05$), a contradiction of Sealander (1943). The presence of minnows was equal for both sexes. Generally, other fish were larger and may have been more difficult to catch. Females apparently tend to feed on smaller prey items, supporting Sealander's (1943) proposal that size of mink may influence size of prey.

CONCLUSIONS

Mink appear to be opportunistic feeders. However, unlike most other predators, they are strictly carnivorous and highly adapted for food gathering in both terrestrial and aquatic habitats. This and other studies suggest that mink do not favor either habitat because terrestrial and aquatic food items are equally prevalent. None of the five major food groups (mammals, fishes, amphibians, birds, and crayfishes) predominated, each varying in importance with season, locality, and year. This flexibility in diet suggests that mink management practices should address diversity; specific management for specifice foods does not seem necessary. Korschgen (1958) believed that sound soil and water conservation practices which

maintain terrestrial and aquatic habitat diversity and quality provide optimum populations of prey species used by mink.

Alteration of wetlands influences their utility as mink habitat and channelization of streams destroys shallow, detritus rich sloughs associated with meandering streams. This elimination of diverse habitats invariably reduces populations of prey species such as crayfish, amphibians, birds, and small mammals. Channelized streams with straight, monotypic banks do not hold flood waters and result in a sharp and minimal transition between aquatic and terrestrial habitats. The impact of impoundment of streams for water storage, flood control, and recreational purposes is widespread; the effect of this activity on mink habitat varies. On streams with low gradation and vast floodplains, an impoundment replaces areas of shallow, highly productive wetlands with deeper water. Only the lake shore remains as an edge between terrestrial and aquatic habitats, resulting in a loss of mink habitat. However, impoundment of streams may yield an increase in shallow water areas by increasing backwaters and flooding timber and meadows. This may enhance mink habitat as the amount of edge between aquatic and terrestrial habitats is increased, creating more desirable areas for production of prey species.

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Table 1. Food items found in digestive tracts of mink collected in southern Illinois during winters of 1974-75 and 1975-76.

Food Item	Number of Occurrences N = 280	Percent Frequency
Mammals	200	71
Rodentia	135	48
Peromyscus spp. (Deer mice)	41	15
Microtus ochrogaster (Prairie vole)	28	10
Mus musculus (House mouse)	20	7
Pitymys pinetorum (Pine vole)	14	5
Ondatra zibethicus (Muskrat)	13	5
Rattus norvegicus (Norway rat)	5	2
Synaptomys cooperi (Bog lemming)	4	1
Oryzomys palustris (Rice rat)	2	1
Zapus hudsonius (Jumping mouse)	2	1
Undetermined rodent	14	5
Lagomorpha		
Sylvilagus spp. (Rabbits)	41	15
Insectivora	7	3
Blarina brevicauda (Short-tailed shrew)	5	2
Cryptotis parva (Least shrew)	1	trace
Undetermined insectivore	1	trace
Undetermined mammal	30	11
Fishes	64	23
Centrarchidae (Sunfish)	19	7
Cyprinidae (Minnows)	16	6
Clupeidae, *Dorosoma* spp. (Shad)	13	5
Catostomidae (Suckers)	10	4
Sciaendae, *Aplodinotus grunniens* (Drum)	2	1
Undetermined fish	12	4
Amphibians	59	21
Anura (Frogs)	25	9
Rana spp. (True frogs)	11	4
Acris spp. (Cricket frogs)	5	2
Undetermined frog	9	3
Amphibian egg masses	5	2
Undetermined amphibian	33	12

Table 1. Continued

Food Item	Number of Occurrences N = 280	Percent Frequency
Crustaceans		
Cambaridae (Crayfish)	39	14
Birds	19	7
Passeriformes	10	4
Icteridae (Blackbirds)	4	1
Pipilo erythrophthalamus (Towhee)	1	trace
Melospisa spp. (Sparrows)	1	trace
Undetermined passerine	4	1
Charadriiformes		
Capella gallinago (Common snipe)	1	trace
Galliformes		
Colinus virginianus (Bobwhite quail)	1	trace
Undetermined bird	7	3
Insects	19	7
Orthoptera (Grasshoppers and crickets)	4	1
Diptera (Fly larvae)	3	1
Hemiptera (True bugs)	1	trace
Hymenoptera (Ants)	1	trace
Coleoptera (Beetles)	1	trace
Megaloptera (Hellgrammites)	1	trace
Undetermined insect	8	3
Spiders	4	1
Reptiles	2	1
Virginia valerae (Earth snake)	1	trace
Undetermined snake	1	trace
Mollusks		
Sphaeriidae (Fingernail clams)	1	trace
Undetermined animal	6	2

Table 2. Comparison of food items found in digestive tracts of male and female mink collected in southern Illinois during winters of 1974-75 and 1975-76.

Food Item	Male		Female	
	No. of Occ. N = 210	Pct. Freq.	No. of Occ. N = 70	Pct. Freq.
Mammals	143	68	59	84
Rodentia	90	43	47	67
Peromyscus spp.	26	12	15	21
Microtus ochrogaster	16	8	12	17
Mus musculus	13	6	7	10
Pitymys pinetorum	10	5	4	6
Ondatra zibethicus	12	6	1	1
Rattus norvegicus	3	1	2	3
Synaptomys cooperi	3	1	1	1
Oryzomys palustris	1	trace	1	1
Zapus hudsonius	1	trace	1	1
Undetermined rodent	11	5	4	6
Lagomorpha				
Sylvilagus spp.	35	17	6	9
Insectivora	4	2	3	4
Blarina brevicauda	3	1	2	3
Cryptotis parva	0	0	1	1
Undetermined insectivore	1	trace	0	0
Undetermined mammal	22	10	7	10
Fishes	57	27	8	11
Centrarchidae	17	8	2	3
Cyprinidae	12	6	4	6
Clupeidae, *Dorosoma* spp.	13	6	0	0
Catostomidae	9	4	1	1
Sciaenidae, *Aplodinotus* sp.	1	trace	1	1
Undetermined fish	11	5	1	1
Amphibians	45	21	13	19
Anura	20	10	5	7
Rana spp.	8	4	3	4
Acris spp.	4	2	1	1
Undetermined frog	8	4	1	1
Amphibian egg masses	5	2	0	0
Undetermined amphibian	25	12	8	11

Table 2. Continued

Food Item	Male		Female	
	No. of Occ. N = 210	Pct. Freq.	No. of Occ. N = 70	Pct. Freq.
Crustaceans				
Cambaridae	29	14	10	14
Birds	14	7	5	7
Passeriformes	6	3	4	6
Icteridae	3	1	1	1
Pipilo erythrophthalamus	0	0	1	1
Melospisa spp.	1	trace	0	0
Undetermined passerine	2	1	2	3
Charadriiformes				
Capella gallinago	1	trace	0	0
Galliformes				
Colinus virginianus	1	trace	0	0
Undetermined bird	6	3	1	1
Insects	13	6	6	9
Orthoptera	3	1	1	1
Diptera	2	1	1	1
Hemiptera	0	0	1	1
Hymenoptera	1	trace	0	0
Coleoptera	0	0	1	1
Megaloptera	1	trace	0	0
Undetermined insect	6	3	2	3
Spiders	2	1	2	3
Reptiles	2	1	0	0
Virginia valerae	1	trace	0	0
Mollusks				
Sphaeriidae	0	0	1	1
Undetermined animal	6	3	0	0

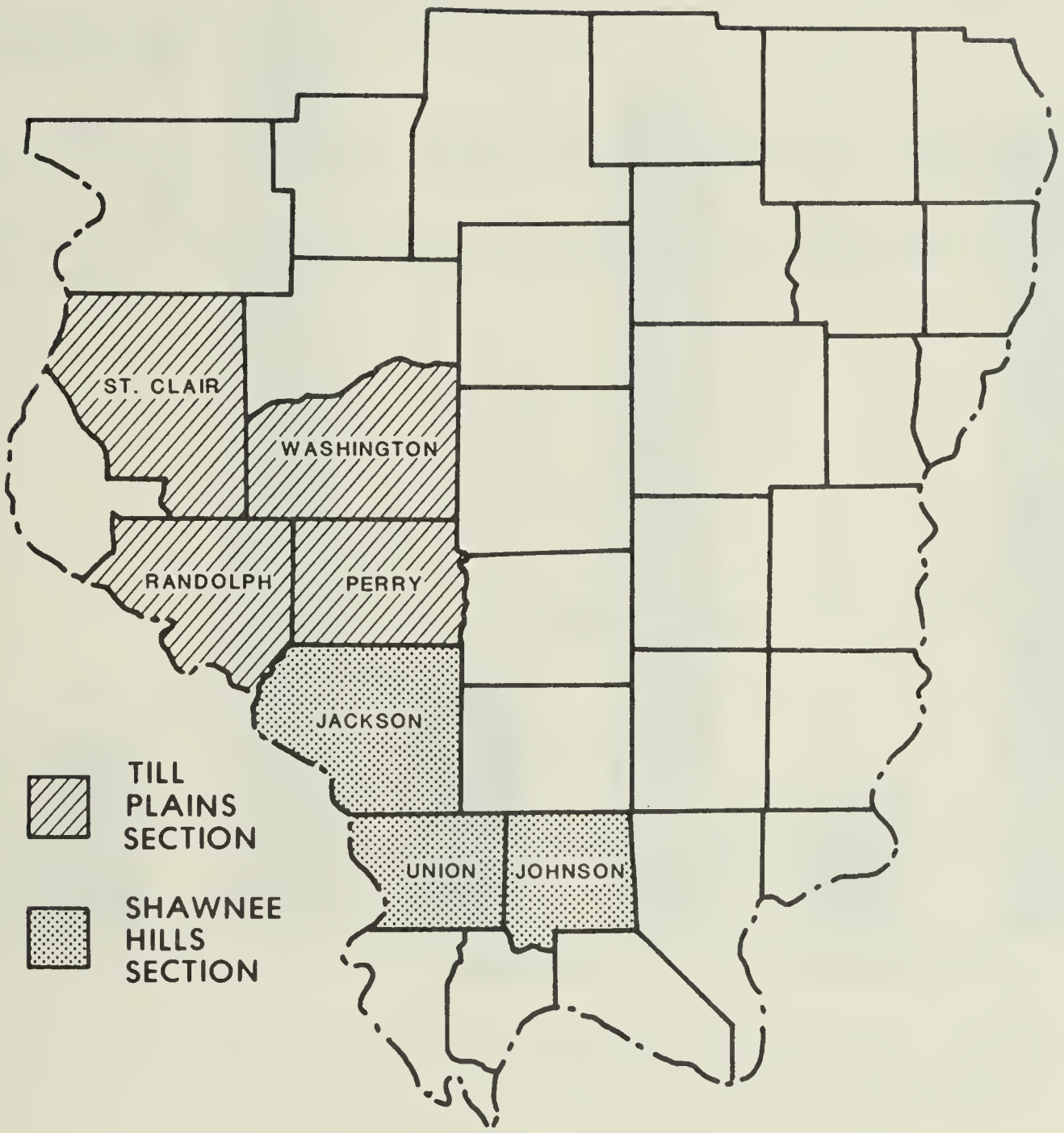

Fig. 1. The study area in southwestern Illinois reflecting the counties where furbuyers and trappers were located and from whom mink carcasses were obtained.

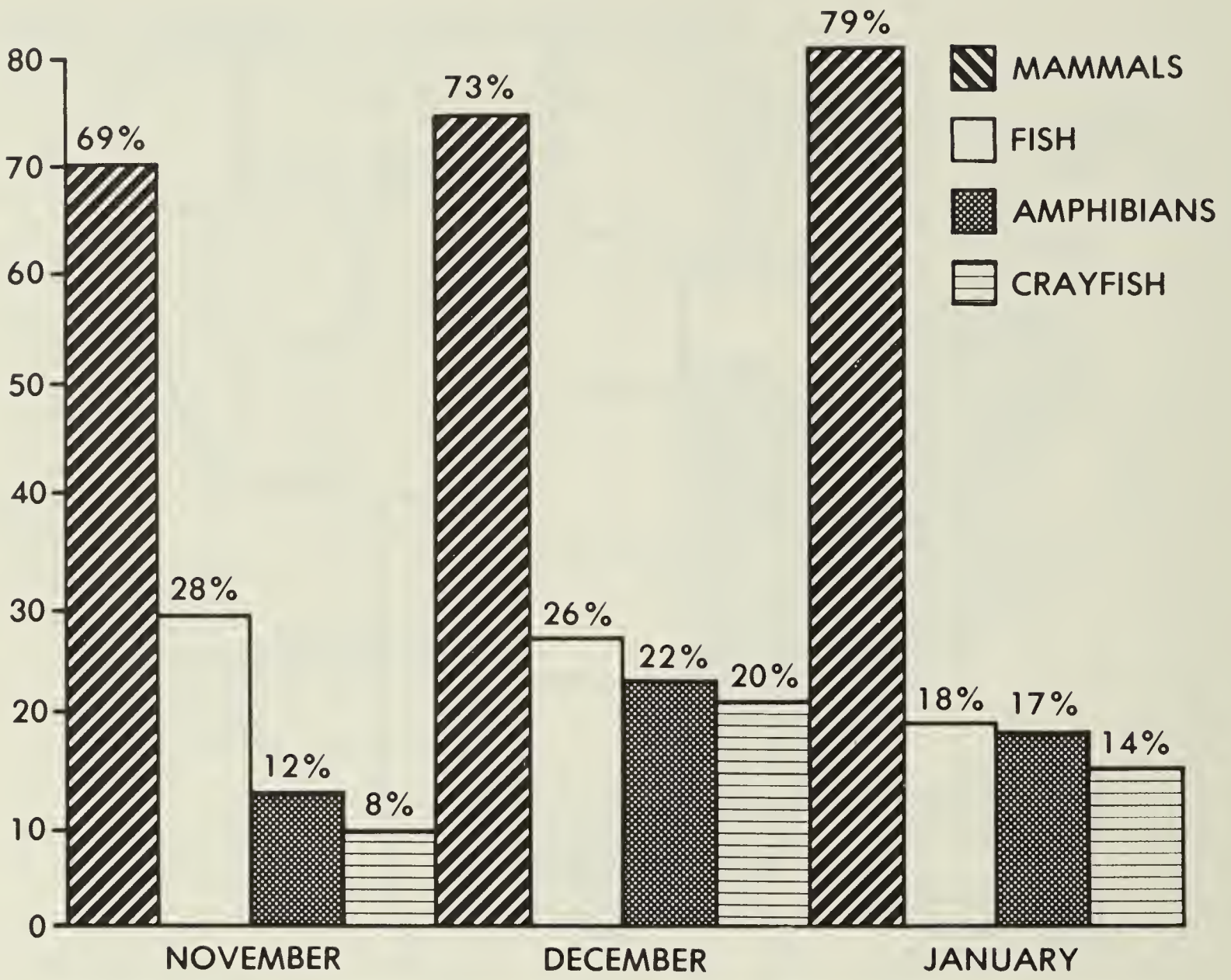

Fig. 2. Monthly trends in percent frequency of occurrence for major food items of 250 minks, southern Illinois, 1974-75 and 1975-76.

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Occurrence of *Oecetis inconspicua* (Walker) in an Acid Strip-Mine Lake of Southern Illinois

Steve Zullo[1]
and
Norman Bates
Department of Zoology
Southern Illinois University at Carbondale
Carbondale, Illinois 62901 USA

ABSTRACT

Zullo, Steve and Norman Bates. (Dep. Zoology, Southern Illinois University, Carbondale, Il 62901, USA). Occurrence of *Oecetis inconspicua* (Walker) in an acid strip-mine lake of southern Illinois.

This study reports for the first time the finding of *Oecetis inconspicua* (Walker) in an acid strip-mine lake of southern Illinois. The caddisfly larvae and pupae were found among *Typha latfolia* detritus in less than one meter of water. Chemical composition of the surrounding water is typically rich in dissolved ions. The pH is around 3.2. Total dissolved solids concentration approaches 2450 mg l^{-1}. The necessity for species identifications in ecological studies is discussed.

INTRODUCTION

Acid strip-mine lakes present conditions that are extreme with respect to several environmental conditions. Many of these lakes have a pH as low as 2.3 (Smith and Frey 1974). Dissolved ion concentration is usually quite high with some lakes in Missouri having concentrations up to 28,500 mg l^{-1} (Campbell and Lind 1969). Metals such as iron, manganese, and aluminum are often found in high concentrations due to increased solubility at low pH (Lewis and Peters 1955, Lind and Campbell 1970, Parsons 1964).

The faunal diversity of acid strip-mine lakes is depressed and typically dominated by few taxa (Smith and Frey 1971). The literature has listed several species of Diptera, Odonata, and Trichoptera as most tolerant of low pH conditions (Bell 1979, Bell and Nebeker 1969, Dills and Rogers 1974, Roback and Richardson 1969).

Species identification is important in all studies, but perhaps it is even more important in studies in acid strip-mine lakes because habitat conditions are vital to the species able to survive there (Resh and Usinger 1975). Many authors however,

[1]Present Address: Department of Chemistry and Biochemistry
Southern Illinois University at Carbondale
Carbondale, IL 62901 USA

do not or cannot identify organisms they find in acid lake studies to the species level. For example, *Chironomus maturus* Johannsen was found in acid conditions (Humphrey 1970), but Yamamoto (1977) later described it as *Chironomus* nomen species based on salivary gland chromosome configuration. Harp and Campbell (1967) identified *C. plumosus* (Linné) from Missouri acid mine lakes; Harp and Campbell (1967) identified *C. plumosus* (Linné) from Missouri acid mine lakes; Harp and Hubbard (1974) subsequently redescribed it as a new species. Roback and Richardson (1969) reported Trichoptera found in acid conditions. They found *Hydropsyche betteni* Ross and *Polycentropus* (probably *Crassicornis*) in Pennsylvania streams polluted with acid mine drainage. Harrison (1948) found *Leptocerus harrisoni* (Barnard) in similar streams. We report here occurrence of *Ocectis inconspicua* (Walker) (Trichoptera: Leptoceridae) in an acid strip-mine lake.

RESULTS AND DISCUSSION

This species was found in Bradley's Acid pit, a 2.3 hectare final-cut acid strip-mine lake located near DeSoto in Jackson County, Illinois. The pH is almost constantly 3.2 and the total dissolved solids concentration is near 2450 mg l^{-1} (unpublished data). The lake has a maximum depth of 8.0 and a mean depth of 3.0 m (Zullo 1981).

Cattails (*Typha latifolia* (Linné)) and Sphagnum moss (*Sphagnum* sp.) comprise the dominant aquatic vegetation. The lake bottom is a mosaic of terrestrial leaves, cattail stalks, Sphagnum moss, and a fine, soft sediment (Zullo 1981).

The water is very clear and has a slight green tint. A Secchi disc (15cm) reading was recorded at the maximum depth (8m) (Bates, personal observation). A thermocline usually starts to develop in April as the water warms. By August, the lake is often homoiothermic and quite warm (27°C). The water cools throughout the fall, developing an inverse thermocline by December. The dissolved oxygen content exhibited a characteristic inverse gradient to temperature in 1977-78 (Zullo 1981). In general, the dissolved oxygen content was high during 1977-78. Concentrations of selected chemical parameters determined by colorimetric methods (Hach 1973), are shown in Table 1.

The fauna of this lake is dominated by a few species. The only zooplankter found is *Brachionus urceolaris* O.F. Muller (Rotifera) (Schramm 1973). The benthos is dominated by *Chironomus* and *Tanytarsus dendyi* Sublette (Diptera: Chironomidae). Another chironomid, *Tanypus nubifer* Coquillett has been collected sporadically (Stahl 1975, Zullo 1981). *Palpomyia* sp. (Diptera: Ceratopogonidae) is common in the littoral zone. The invertebrate predators include naiads of the odonates *Anax junis* (Drury) and *Enallagma*, larvae or the trichopteran *Oecetis inconspicua* (Walker), and *Palpomyia* sp. larvae. There are no fish present.

TRICHOPTERA COLLECTIONS

The dates of collection, number, and head capsule width (mm) of specimens of *O. inconspicua* (Walker) collected are presented in Table 2. All specimens were collected with an Ekman grab (225cm^2) or a coarse mesh dipnet. The larvae were collected exclusively around the cattail and leaf detritus at the northeast corner of the strip pit in a meter or less of water. Live larvae were brought back to the labo-

ratory, but none pupated. One pupa brought back eventually emerged to an adult male. Identification was based on the description in Ross (1944).

Ross (1944) reported that *O. inconspicua* is one of the most common caddisflies in Illinois, taken throughout the state from a diversity of habitats. However, to our knowledge these are the first collections from an acid strip-mine lake in Illinois.

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Table 1. Surface in Water Chemistry in Bradley's Acid Pit in 1977-78. In mg l^{-1}.

Date	Al^{3+}	Total Fe	Mn^{2+}	PO_4^{3-}	SO_4^{2-}
23 Aug 77	26.5	5.0	—	62.5	1850
23 Sep	26.5	4.6	28.0	53.5	1950
27 Oct	22.5	4.4	40.0	55.0	1525
21 Nov	22.5	5.3	20.0	60.0	1450
19 Dec	23.5	5.5	20.0	56.0	1475
21 Jan 78	26.0	6.9	21.0	44.5	1400
24 Feb	19.0	4.2	12.0	27.5	1300
22 Mar	12.0	3.0	11.8	29.0	800
26 Apr	21.5	13.0	120.0	87.5	1350
24 May	24.0	4.9	—	42.0	1350

(Modified from Zullo 1981).

Table 2. Head Capsule Widths of *Oecetis inconspicua* larvae collected in Bradley's Acid Pit in 1978 and 1981.

Head Capsule Width (mm)	Collection Date 19 Apr 78	3 May 78	19 May 78	26 Jul 81
0.20	1			
0.35	1			
0.42				1
0.44				1
0.47	1			
0.48				1
0.52			1	
0.70				1
0.72				3
0.74				1
0.75			1	1*
0.76			1	
0.78		1		
0.82		1		

*Was in pupal case.

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Work Energy in a Final-Cut Strip Mine Lake in Southern Illinois

by
Michael J. Chimney
Cooperative Wildlife Research Laboratory
and
Department of Zoology
Southern Illinois University
Carbondale, Illinois 62901

ABSTRACT

The work parameters: Birgean Work (B), Schmidt Stability (S), and Total Direct Work (G), were calculated for a final-cut strip mine impoundment located in southern Illinois using density data compensated for 1) temperature (= uncorrected work parameters: B, S, G) and 2) temperature, pressure, and salinity (= corrected work parameters: B′, S′, G′). Maximum work energies ocurred after the summer solstice and were comparable to levels observed in similar holomictic lakes. In general, $B' < B, S' > S$, and $G' < G$; These differences were attributed mainly to salinity; pressure was less important. Corresponding corrected and uncorrected work parameters were highly correlated with, and at the same time statistically different from, each other. Only differences between B′ and B were regarded as limnologically significant however.

INTRODUCTION

Prior to the enactment of federal legislation (P.L. 95-87), surface coal mines were often abandoned unreclaimed at the end of mining operations. This practice left areas of highly disturbed topography that easily collected water, creating thousands of freshwater strip mine lakes and ponds nationwide. Impoundments which formed in final-cut pits are often morphologically, chemically, and biologically distinct from lentic systems of other origins. However, despite their abundance and unusual characteristics, few final-cut strip mine lakes have been intensively studied, especially with respect to their physical limnology.

Calculation of the work parameters: Birgean Work, Schmidt Stability, and Total Direct Work (Birge 1916; Schmidt 1915, 1928), provides useful comparative data on the lacustrine energy dynamics within a lake (Hutchinson 1957, Johnson et al. 1978). Yet, these parameters seldom have been employed as working limno-

logical tools (Johnson et al. 1978). There are no work energy data available for final-cut strip mine impoundments, nor were the raw data from published studies sufficient to permit accurate computation of these parameters. To this end, I will present an analysis of work energy within such a system located in southern Illinois.

METHODS

This study was conducted on Moroni's Big Lake (MBL), a dimictic final-cut impoundment (5.47 ha) located in Jackson County, Illinois. A description of the study area can be found in Chimney (1982). Data necessary to compute the work parameters were collected May to December 1978 and March to December 1979 at a single open-water station located in the deepest portion of MBL (Chimney 1982, Fig. 1). Temperature and specific conductance measurements were recorded at 1 m intervals (0-13 m) using the thermistor on a YSI 57 oxygen meter and a Barnstead DM-70 CB conductivity bridge. All specific conductance values were corrected to 25°C.

Birgean Work is defined as the energy expended by the wind in creating the observed density distribution starting with an initial condition of isothermy and minimum heat (generally 4°C) (Cole 1979, Idso 1973):

$$B = A_o^{-1} \int_o^{z_m} z(p_i - p_z) A_z \, dz \qquad (1)$$

where B = Birgean Work (g-cm cm^{-2}), A_o = surface area (cm^2), A_z = surface area at depth z (cm^2), z = depth (cm), z_m = maximum depth (cm), Pi = initial density (g cm^{-3}), and p_z = observed density at depth z (g cm^{-3}). Schmidt Stability is the subsequent work that would have to be performed on the observed density structure to overcome stratification and mix the lake to uniformity without adding or substracting heat in the process (Cole 1979, Idso 1973):

$$S = A_o^{-1} \int_o^{z_m} (p_z - \bar{p}) A_z (z - z_{\bar{p}}) \, dz \qquad (2)$$

where S = Schmidt Stability (g-cm cm^{-2}), $\bar{p}$ = final density attained upon mixing the lake to uniformity (g cm^{-3}), and $z_{\bar{p}}$ = depth where $\bar{p}$ exists prior to mixing (cm). Total Direct Work is the sum of these two parameters for a given density distribution (Hutchinson 1957):

$$G = B + S \qquad (3)$$

where G = Total Direct Work (g-cm cm^{-2}). Equations 1 and 2 were evaluated using standard numerical analysis techniques (Adams 1963, McCarty 1975).

Discussions of work energy in limnology texts are usually found in sections dealing with heat. This is because limnologists commonly regard the density of freshwater as a function of its temperature, ignoring other factors such as pressure and salinity. While this is a valid approach in many situations, it is known that at even moderate levels of pressure (> 1.0 bar) or salinity (> 0.15 ppt), the actual density of water differs appreciably from values based solely on temperature (Chen and Millero 1977). Only studies of meromictic lakes (e.g. Effler et al. 1981, MacIntyre and Melack 1982, Walker 1974) have, to date, corrected density for

pressure/salinity before work parameters were calculated. The importance of pressure/salinity to work energy in MBL was examined by deriving two separate density distributions for each date. First, "uncorrected" densities were computed from temperature data using the equation of Tilton and Taylor (1937). Second, "corrected" densities, compensaterd for temperature, pressure, and salinity, were derived following Chen and Millero (1977). Salinity was estimated from specific conductance values (Kemp 1971). Subsequently, "uncorrected" (designated B,S,G,) and "corrected" (designated B′,S′,G′) work parameters were computed using the appropriate density estimates.

All statistics were calculated using SAS (SAS Institute 1982). The critical level of significance (α) was set at 0.01 in all cases.

RESULTS AND DISCUSSION

Maximum work energies in MBL (Table 1) were comparable to values reported for other strongly stratified holomictic lakes of similar size and depth (cf. Hutchinson 1957, Johnson et al. 1979). All work parameters reached their maximum values after the summer solstice (Fig. 1). This would be expected as MBL gained an appreciable proportion of its summer heat budget (21.4% and 18.8% in 1978 and 1979) between this date and the end of the heating period (the date of maximum mean temperature) (Chimney 1982).

Throughout the study, $B' < B$ (Fig. 1). Maximum Birgean Work was achieved as MBL began to cool and other measures of lake energy (e.g. mean temperature, Schmidt Stability, Total Direct Work) were on the decline. However, until stratification was completely destroyed, heat continued to be transported down through the metalimnion. Net heat gain in the meta- and hypolimnion apparently more than offset epilimnetic loss during the initial stages of turnover, resulting in the continued increase in Birgean Work for a short period of time.

Densities in the lower hypolimnion, when corrected for pressure/salinity, often actually increased relative to p_i (see equation 1). This resulted in negative values of Birgean Work for these strata. It is suggested that no wind-related work was done at these depths; rather, density currents, produced biogenically (Stahl 1979), were responsible for heat transport at these times.

Changes in Schmidt Stability were highly correlated with thermal variation in the 0-1 m stratum of MBL (Table 2) with maximum values for this parameter occurring on the dates of highest surface temperatures (except S′ in 1979) (Fig. 1). Correcting density for pressure/salinity generally resulted in $S' > S$. Note however, that from June to August 1979 the opposite was true.

Variability in Total Direct Work closely paralled changes in mean lake temperature (Table 2). The dates of maximum Total Direct Work also marked the maximum mean lake temperature (Fig. 1) and the highest summer heat budget (Chimney 1982). Overall, levels of Total Direct Work, when corrected for pressure/salinity, were less than those based on temperature profiles alone.

Differences between corresponding corrected and uncorrected work parameters (Fig. 1) were attributed mainly to the influence of salinity; pressure was thought to be of minor importance. Salinity ranged from 1.5 to 2.1 ppt (i.e. 2294 to 3136 μmhos cm^{-1}) (Fig. 2), levels an order of magnitude greater than the critical value of Chen and Millero (1977) and typical of other strip mine lakes in the region (cf. Lewis and Peters 1954, McConathy and Stahl 1982). By comparison, only $\sim$ 10% of total lake volume was at a pressure $>$ 1.0 bar.

Corresponding corrected and uncorrected work parameters, while highly correlated (Table 3), were statistically different from each other with the exception of Schmidt Stability in 1979 (Table 4). However, only the differences between corrected and uncorrected Birgean Work were felt to be limnologically significant in terms of accurately documenting the lacustrine energy dynamics of MBL.

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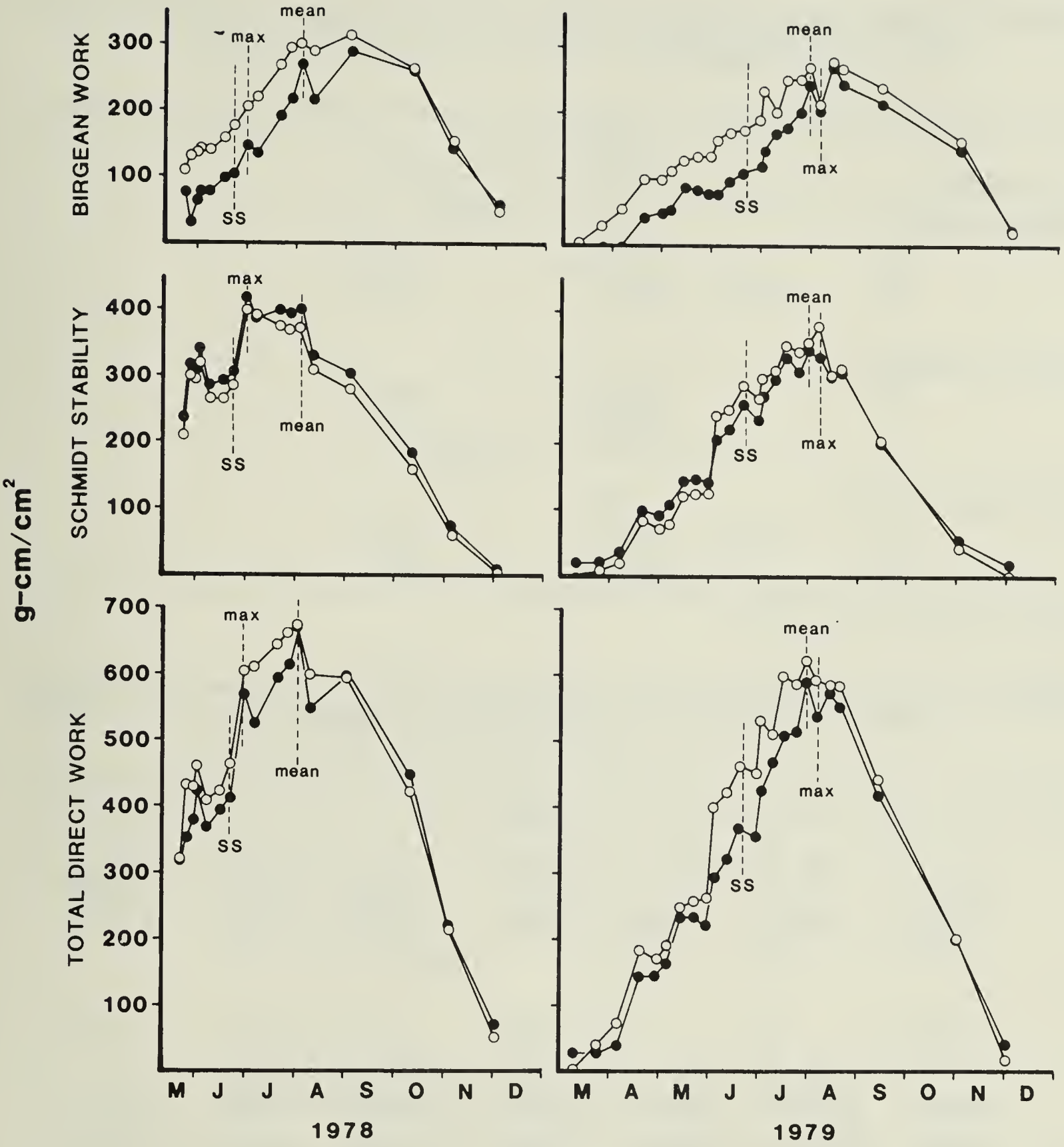

Fig. 1. Birgean Work, Schmidt Stability, and Total Direct Work in Moroni's Big Lake. (•) = values corrected for pressure/salinity; (o) = values uncorrected for pressure/salinity; (SS) = date of summer solstice; (mean) = date of maximum mean lake temperature; (max) = date of maximum surface temperature.

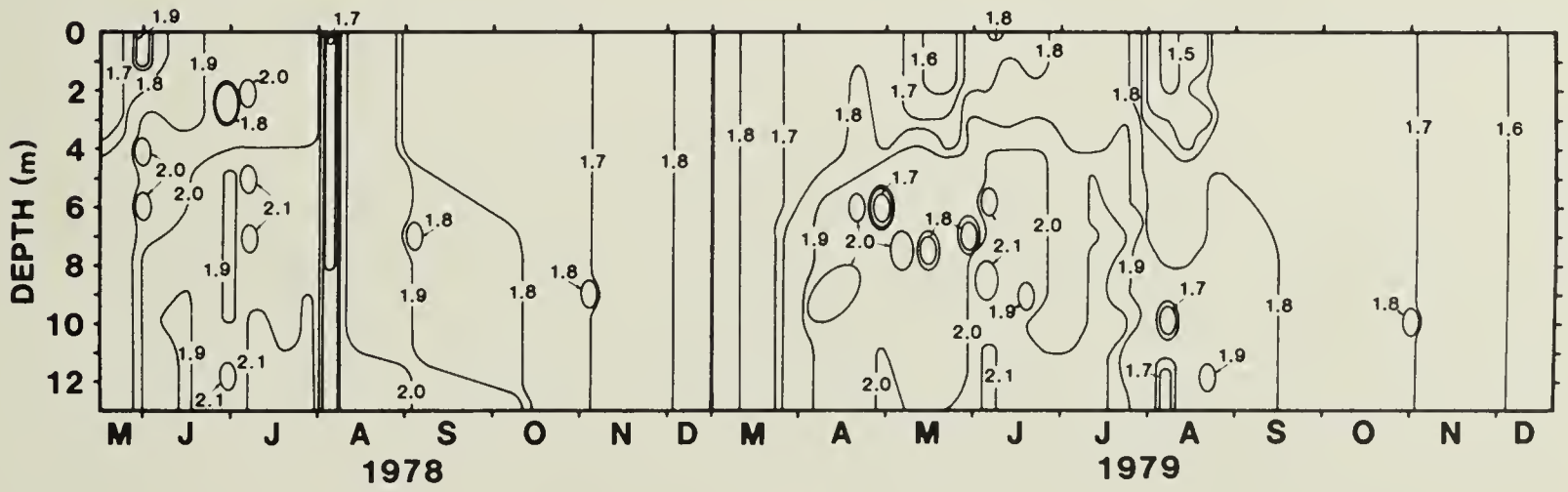

Fig. 2. Time-depth diagram of salinity (ppt) for Moroni's Big Lake.

Table 1. Maximum Birgean Work, Schmidt Stability, and Total Direct Work from Moroni's Big Lake.*

Parameter	Year 1978	1979
Birgean Work		
B	316.3	281.8
B′	289.6	273.3
Schmidt Stability		
S	398.0	380.8
S′	420.5	346.1
Total Direct Work		
G	675.9	627.2
G′	673.4	592.3

*All values expressed as g-cm cm^{-2}.

Table 2. Pearson product-moment correlations of work parameters with surface and mean lake temperature from Moroni's Big Lake.

	1978					
	B	B′	S	S′	G	G′
surface	ns	ns	0.9747	0.9795	0.9216	0.8616
mean	0.9228	0.7316	0.8008	0.8142	0.9658	0.9767
	1979					
	B	B′	S	S′	G	G′
surface	0.9104	0.7914	0.9580	0.9618	0.9621	0.9240
mean	0.9713	0.8780	0.9235	0.9383	0.9645	0.9481

Table 3. Pearson product-moment correlations between corrected and uncorrected work parameters from Moroni's Big Lake.

	1978		
	B′	S′	G′
B	0.9262		
S		0.9979	
G			0.9829

	1979		
	B′	S′	G′
B	0.9478		
S		0.9939	
G			0.9855

Table 4. Paired comparison t-tests between corrected and uncorrected work parameters from Moroni's Big Lake.

Year	Parameter	T	df	PR > T
1978	Birgean Work	6.92	23	0.0001
	Schmidt Stability	-11.39	23	0.0001
	Total Direct Work	3.89	23	0.0013
1979	Birgean Work	7.90	16	0.0001
	Schmidt Stability	0.18	16	0.8621
	Total Direct Work	5.40	16	0.0001

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Regional Variation in Metals in Livers of White-Tailed Deer in Illinois[1]

Alan Woolf, Catherine Gremillion-Smith, and Charles O. Frank[2]
Cooperative Wildlife Research Laboratory
Southern Illinois University
Carbondale, Illinois 62901

ABSTRACT

Concentrations of nine heavy metals found in livers of white-tailed deer (Odocoileus virgineanus) in Illinois exhibited significant regional variation. The findings illustrated both the utility of white-tailed deer to monitor environmental quality and also the need for at least regional, and perhaps site specific surveillance. Grouping on a regional ecological basis was useful for surveillance of Co, Cr, Cu, Mg, Mn, Ni, Pb, and Zn. Cadmium had a greater variability in distribution within the regional groupings suggesting that more site specific monitoring is needed for this trace element.

INTRODUCTION

Woolf et al., 1982 reported heavy metal concentrations found in liver samples from white-tailed deer collected from 15 areas of Illinois. The utility of using deer for such monitoring was shown and the baseline data were useful for comparison with other published findings. However, retrospectively there appeared to be a need to further define the geographical variation within Illinois based on a larger sample. Hence, more data were collected with the objectives of: 1) determining heavy metal concentration variability between distinct geographical regions in Illinois; and 2) identifying areas of the state for proposed monitoring in time and space.

MATERIALS AND METHODS

Liver tissue was collected from 441 white-tailed deer in 1980 and 1981 harvested in 26 counties plus Crab Orchard National Wildlife Refuge (CONWR) in William-son County (Fig. 1). All but 32 samples were obtained from hunters at

[1]Supported by Illinois Department of Conservation Federal Aid to Wildlife Restoration Project W-63-R (SI) VI.

[2]Department of Geology, Southern Illinois University at Carbondale, Carbondale, Illinois 62901.

mandatory deer check stations; the remainder were collected from road-killed deer by cooperators in the west-central Illinois region. Samples were placed in plastic "whirl-pacs" and identified by county of collection; specific site of harvest was unknown. Samples were frozen within 12 hours then transported to the laboratory and stored in a freezer (– 10C) until processed for analysis. Samples from CONWR were handled similarly except hunters were interviewed to determine kill locations which were plotted on a 7.5′ topographic map.

Methods of collection did not permit choice of liver tissue nor did they include safeguards against contamination. During laboratory processing, a 4-6 g subsample was taken by trimming away superficial areas with a scalpel. Samples were then digested with nitric and sulfuric acids; blanks of all chemicals were processed concurrently with tissue digestions and were used to determine levels of background contamination. A Jarrell-Ash Model 975 Plasma Atom Comp Direct Spectrometric System was used to analyze digestates and the blank matrices as previously described (Woolf et al, 1982).

Data were grouped into four regions for analysis; CONWR was treated as a separate region although it is located in southern Illinois. The assignment of counties to regions was based on Illinois deer management units and approximated natural ecological divisions identified by Schwegmen (1973). Sex and age classes were grouped for analysis so sample sizes would permit statistical analysis at the county level. The General Linear Models Procedure in the SAS package (Helwig and Council, 1979) and Southern Illinois University at Carbondale computing facilities were used to do the ANOVA and Duncan's Multiple Range Test which would detect mean differences of each element within and between the four groupings. Levels of significance for all tests were $P < 0.05$.

RESULTS

Significant regional differences were found in mean liver concentrations (μ g/g dry wt.) for all nine elements evaluated (Table 1). CONWR had the highest concentration of every element except Co and Mg. In the case of Co, the mean was not significantly different than that in the region with the highest concentration.

The validity of the regional groupings was examined by assessing intraregional variation statistically. The 15 counties grouped as west central Illinois did not have significant differences in liver levels of Mg, Ni, Pb, or Zn. Significant differences existed in Co, Cu, and Mg levels, but 12, 14, and 13 counties, respectively, were similar.

There was significant variation of Cd levels within the region suggesting that this element requires more site specific surveillance than afforded by the regional grouping. Seven counties were similar with levels ranging from 0.13-0.28 ppm; eight others were similar with smaller concentrations from 0.01-0.12. Further, there was overlap in the mid-range with six counties not significantly different.

The northern and southern Illinois regions were more uniform than the west central region. Significant intra-regional differences were found only for Co, Ni, and Zn in southern Illinois; in all cases, the same four of five counties were similar.

DISCUSSION

These data illustrate the importance of determining regional variability and considering such in any geographical monitoring scheme. The baseline data pre-

viously reported by Woolf et al (1982), while useful for comparison with metal concentrations reported for deer or similar species in other areas, were biased by a large proportion of samples (69%) from CONWR. Likewise, the mean values in this study (Table 1) are biased if viewed as a group rather than regionally because CONWR data comprises 34% of the total sample. The necessity for regional monitoring is apparent. In the case of Cd, the intra-regional variation suggests the need for even more site specific monitoring.

Data on intra-regional variation must be evaluated with caution because of the uneven, and sometimes small sample sizes (Figure 1). Further, sex and age classes were combined for these analyses although Woolf et al. (1982) and others have noted sex and/or age influences on tissue concentrations of some metals. We accepted this potential additional source of variation because there was no significant difference in the age-structure (fawn, yearling, and 2 1/2 year old and older) of the samples representing each region ($X^2 = 5.61$ w/6 d.f.; $P < 0.50$). While there was a significant difference ($X^2 = 9.88$ w/3 d.f.; $P < 0.02$) in the sex structure of the samples (50.5, 54.8, 38.6 and 68.9% males for CONWR, west central, southern, and northern Illinois regions, respectively) these may have influenced only Cr, Cu, and Mg levels (Woolf et al. 1982). Also, the magnitude of differences between regions far exceeded those previously noted between sexes. However, we do recommend that this potential source of experimental variation be eliminated by limiting sampling to a single sex/age class.

The need to monitor environmental levels of heavy metals in time and space is well established and the regional differences found in this study illustrate the utility of such efforts. For example, CONWR samples had comparatively high concentrations of most metals. Especially noteworthy were the levels of Cd and Pb which are among those elements reported to constitute the greatest hazard to human health (Jenkins, 1981). The Cd levels at CONWR are nearly two-fold greater than elsewhere in southern Illinois, and about 4.5 times greater than other regions. Lead levels at CONWR ranged from 2.6 to 18.6 times greater than other regions (Table 1). While we do not imply that any hazard exists, clearly the findings indicate the value of such data, especially if the findings are then followed by an effort to determine the sources, actual extent, and biological significance of given levels of heavy metals in the environment.

The geographical dispersion of trace metals and the natural and man induced factors that influence flow and storage in ecosystems clearly need definition and understanding. Baseline data as presented identify status and provide a useful basis for further study. White-tailed deer, a widely distributed species, are useful for broad monitoring that can then form the basis for more site specific studies focused on soils, plants, and more sedentary animals.

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Fig. 1. Collection sites for livers of white-tailed deer, 1981-82. Numbers of livers collected from each area are as follows: (1) Jo Daviess Co., n = 21; (2) Boone Co., n = 4; (3) McHenry Co., n = 5; (4) DeKalb Co., n = 11; (5) Rock Island Co., n = 15; (6) Henry Co., n = 3; (7) Mercer Co., n = 5; (8) Henderson Co., n = 6; (9) Warren Co., n = 14; (10) Knox Co., n = 8; (11) Peoria Co., n = 8; (12) Marshall Co., n = 12; (13) Woodford Co., n = 7; (14) Tazewell Co., n = 33; (15) Fulton Co., n = 30; (16) McDonough Co., n = 16; (17) Schuyler Co., n = 2; (18) Hancock Co., n = 4; (19) Adams Co., n = 8; (20) Pike Co., n = 10; (21) Washington Co., n = 3; (22) Jackson Co., n = 2; (23) Williamson Co., n = 29; (24) Saline Co., n = 4; (25) Union Co., n = 19; Crab Orchard National Wildlife Refuge, n = 151.

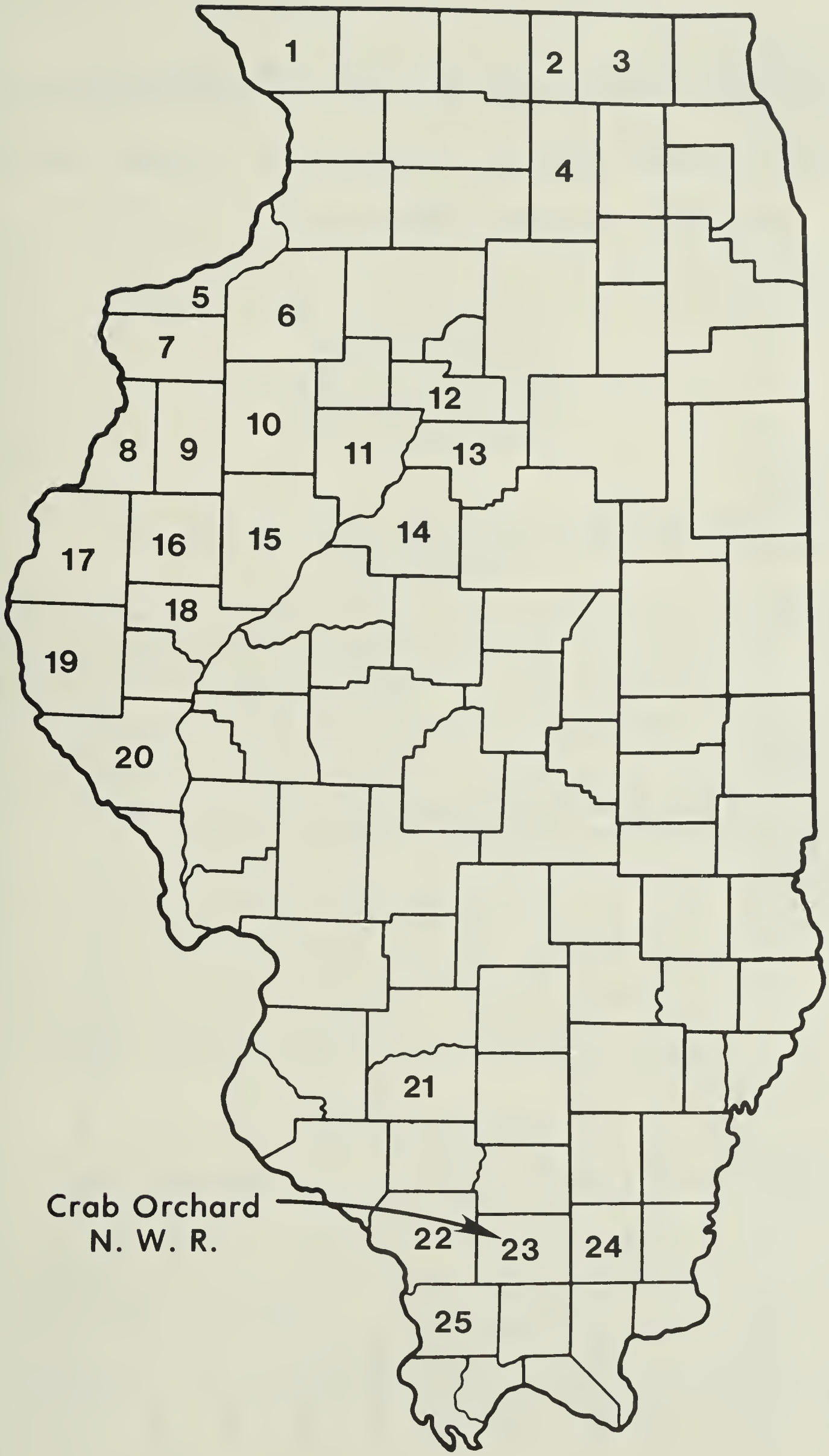
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Table 1. Regional mean trace metal content of Illinois white-tailed deer liver tissue, 1980 and 1981 (ug/g dry wt.).

Element	Total (n = 441) $\bar{X}$ ± SE	Crab Orchard[a] NWR (n = 151)	W. Central[b] Ill. (n = 188)	S. Ill.[c] (n = 57)	N. Ill.[d] (n = 45)
Cadmium	0.23 ± 0.02	0.43 (a)[e]	0.10 (c)	0.25 (b)	0.09 (c)
Cobalt	0.20 ± 0.02	0.36 (a)	0.05 (b)	0.45 (a)	0.04 (b)
Chromium[f]	2.7 ± 0.1	3.1 (a)	2.3 (b)	1.0 (c)	
Copper	76 ± 3	115 (a)	56 (b)	49 (b)	55 (b)
Magnesium	206 ± 3	211 (b)	212 (b)	154 (c)	235 (a)
Manganese	5.9 ± 0.2	8.4 (a)	5.0 (b)	3.9 (b)	4.0 (b,c)
Nickel	2.5 ± 0.3	4.5 (a)	1.7 (b)	0.8 (b)	1.0 (b)
Lead	2.8 ± 0.2	5.6 (a)	1.3 (b,c)	2.1 (b)	0.3 (c)
Zinc	65 ± 2	69 (a)	61 (a,b)	55 (b)	67 (a)

[a]Crab Orchard National Wildlife Refuge located in Williamson Co., Southern Illinois
[b]15 counties represented
[c]5 counties represented
[d]6 counties represented
[e]Means with the same letter are not significantly different (Duncan's Multiple Range Test)
[f]Chromium only tested for in 1980 samples, n = 205

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Geologic History of the Mackinaw Member of the Henry Formation in the Illinois River Valley in Tazewell County, Illinois

Thomas K. Searight
Department of Geography-Geology
Illinois State University
Normal, IL 61761

ABSTRACT

In the Illinois River valley the Mackinaw Member of the Henry Formation consists of outwash gravels and sands of Woodfordian (Wisconsinan) age. Although the earliest outwash is buried, the upper 50 to 75 feet are exposed in several areas. The following history is based on bedding structures, lithologies of clasts, and textures. The lowest exposed units are planar cross-bedded clean quartz sands with a few scattered pebbles. A series of gravels with prominent scour and fill cross-bedding that contain large clasts of black shale, coal, and armored mudballs lie on the eroded surface of these sands. Flat-bedded sandy gravels devoid of black shale and coal succeed these deposits. These three series of sediments indicate initial low water velocities followed by a sudden rapid increase to very high velocities and a final decrease to moderate velocities. Floodplain alluvium rests on the flat-bedded gravels. A great flood swept over the floodplain, eroded broad channels, removed all fine material, and left an aggregate of rounded cobbles and boulders. Prominent lithologies among these lag deposits are black shale, coal, and armored mudballs. Floodplain alluvial deposition resumed as the flood waned.

INTRODUCTION

The Henry Formation, named for Henry, Marshall County, (Willman and Frye, 1970) consists of sand and gravel outwash deposits that are Woodfordian (Wisconsinan) in age. On the basis of lithology and origin, the formation is subdivided into three members, the Batavia, the Wasco, and the Mackinaw. Outwash plains are included in the Batavia, ice contact deposits comprise the Wasco, and valley trains make up the Mackinaw. The deposits in the Illinois River valley in Tazewell County make up a portion of a large valley train that are included in the Mackinaw Member, named for Mackinaw, Tazewell County (Willman and Frye, 1970).

In the Illinois River valley in Tazewell County only the upper 50 to 75 feet of the Mackinaw Member are exposed in surface outcrops, and, therefore, this discussion is restricted to this portion of the valley train. Concentrations of lithologic types, sizes of particles, and primary bedding structures were found to be the best keys to the depositional history of the Mackinaw Member.

PREVIOUS WORK

The sand and gravel deposits in Tazewell County have been described in a number of previous investigations. However, most of these are either regional studies that cover the geology of the county in a general way or deal with the economic aspects of the deposits rather than the depositional history of them. Bannister (1870) discussed the geology of west-central Illinois and included the floodplain gravels along the Illinois River. Leverett (1899) included Tazewell County in his description of the deposits of the Illinois glacial lobe. The northern part of the county was covered by Barrows (1910) in his geographic study of the Middle Illinois valley. The geology and mineral resources of that portion of Tazewell County within the Peoria 15′ Quadrangle was discussed by Udden (1912). Horber (1950) and Piskin (1967) discussed the bedrock valleys in the county and described the sand and gravel deposits that fill them. Horberg *et al.* (1950) and Walter *et al.* (1965) discussed the sands and gravels of parts of the county as sources of ground water. Wanless (1957) described the deposits in the Illinois River valley in Mason and Tazewell Counties and interpreted the geologic history of the area. The sand and gravel deposits of the county were studied in detail as potential economic resources by Hunter (1966). Willman and Frye (1970) classified the Pleistocene deposits in Illinois and established the formal stratigraphic nomenclature used throughout the state. Willman (1973) summarized the stratigraphy and geologic history of the Illinois Waterway including Tazewell County.

BEDROCK GEOLOGY

The prePleistocene surface in Tazewell County is dominated by the broad valleys of the ancestral Mississippi River, the Mackinaw Bedrock valley, and the Teays River, the Mahomet Bedrock valley (Figs. 1 and 2). Intervalley areas are restricted to several hills that form islands between the valleys. Exposures of bedrock are restricted to a few small stream cuts in the western part of the county (Fig. 2). The preglacial valleys were filled with Pleistocene tills and outwash and have no surface expression in the eastern part of the county.

The modern Illinois River has established its channel in part on fill within the bedrock valleys and in part on glacial deposits that covered the bedrock upland west of the valleys. Above Peoria the river flows along the west side of the broad Mackinaw Bedrock valley. Between Peoria and Pekin the valley has been incised into bedrock, producing the narrow Pekin-Sankoty Channel. Below Pekin the channel is along the west side of the broad valley below the confluence of the ancestral Mississippi and Teays Rivers (Fig. 2). Thus the width and character of the modern Illinois River valley is controlled by the type of material into which it is eroded.

In this study two segments of the Illinois River valley are of importance. The upper segment, the Pekin-Sankoty Channel, is cut into typical Pennsylvanian cyclic strata, producing a narrow rock-walled valley approximately one and one half

miles wide. In the lower segment the valley widens to as much as twenty miles in the southwestern portion of the county. The western boundary of the valley is a series of bluffs that, like the narrows above, are Pennsylvanian bedrock. The eastern bedrock valley wall is the buried eastern limit of the Mackinaw Bedrock valley so that the eastern side of the modern Illinois River valley is composed of Pleistocene sediments. These relationships are illustrated in Figure 3.

SURFICIAL GEOLOGY

With the exception of the few small exposures shown in Figure 2, the bedrock surface in Tazewell County is covered by glacial and glacial-fluvial deposits (Figs. 3 and 4). Uplands are dominantly Illinoian till plains overlain by Wisconsinan till plains, end moraines, and loess (Fig. 4). The Illinois River valley is partially filled with a series of gravel and sand outwash deposits overlain by alluvium along the Illinois and Mackinaw Rivers and dune sands elsewhere (Figs. 3 and 4). The Mackinaw Member of the Henry Formation is a complex valley train that makes up the youngest outwash deposits in the valley fill. Wanless (1957, Fig. 58) reconstructed the original surface of the valley train. He estimated elevations of 640 feet at the proximal end near Peoria and 480 feet at the distal end in southwestern Mason County. The maximum valley fill (Fig. 5) was calculated from the differences in elevations between the bedrock surface (Fig. 2) and the original surface of the valley train constructed by Wanless (1957). Valley fill is thickest in two areas. Deposits more than 240 feet thick fill the old Mackinaw-Mahomet Bedrock valleys. However, these deposits are not aligned with the only Wisconsinan drainage in the vicinity, the modern Mackinaw River valley, and, therefore, must be largely older than the Henry Formation. The second area of thick outwash is in and below the Pekin-Sankoty Channel. Here sediments exceed 220 feet in thickness and are in the primary site of deposition of the Henry Formation (Willman and Frye, 1970, and Willman, 1973). Surface outcrops expose only the upper 50-75 feet of Wisconsinan sediments so the stratigraphic sequence of the entire valley fill cannot be determined. Based on well records, Horberg (1950) indicates that at least the lower portion of the valley fill is preWisconsinan in age.

The original surface of the Mackinaw Member has been greatly reduced by erosion to a series of three terraces (Fig. 4). Wanless (1957) designated the terraces, the Manito, Havana, and Bath named for towns in the area. The highest terrace, the Manito, ranges in elevation from approximately 540 feet in the north to approximately 510 feet in the south. The intermediate terrace, the Havana, is slightly lower than the Manito and ranges in elevation from approximately 500 feet in the north to 480 feet in the south. The lowest terrace, the Bath, lies between the modern floodplain of the Illinois River and the Havana terrace and averages 470 feet in elevation.

The youngest sediments deposited on the surface of the valley train are wind deposited dune sands and modern stream alluvium and peat. The dunes are most abundant on the Manito terrace, but are also common on the Havana terrace and on the western borders of the uplands to the east beyond the limits of the valley fill. Modern alluvium has been deposited by both the Illinois and Mackinaw Rivers (Fig. 4). Peat beds of limited areal extent occur on the floodplain of the Mackinaw River.

PETROLOGY

The Mackinaw Member is composed of interbedded gravels and sands. Although the gravels consist of clasts that range in size from pebbles as small as five millimeters to boulders several meters in diameter, pebbles and small cobbles are most abundant. Lithologically the gravels are highly variable, and all of the major rocks groups, sedimentary, igenous, and metamorphic, are present. Limestones, dolomites, sandstones, and granitic igneous clasts are the most common varieties. However, black shales, coals, and armored mudballs are locally very abundant near the head of the valley fill. The largest boulders occur in and near the Pekin-Sankoty Channel. Down valley there is a rapid decrease in the sizes of the largest clasts so that near the southern border of the county only small to medium pebbles are found.

The sand deposits as well as the sand fractions in the gravels are composed predominantly of quartz. Other minerals present in measurable quantities derived from igneous, sedimentary, and metamorphic terraines are typical of deposits related to the glaciation of Illinois. Willman and Frye (1970) list the complete mineralogy of the sands in the Henry Formation. Measurements of the median and mean diameters of the sands indicate a progressive decrease in grain sizes down valley. Thus measurements of both the largest clasts and sands indicate a loss of current velocity away from the source of the valley train.

Primary bedding structures are good indicators of the relative velocities of the currents that transported and deposited the sands and gravels. Several gravel pits in and near the proximal portion of the valley train expose bedding in the upper 50 to 75 feet of outwash. Scour and fill cross-bedding is the most common structure, but planar cross-bedding and flat-bedding are also present.

DEPOSITIONAL HISTORY

The most important keys to the depositional history of the Mackinaw Member of the Henry Formation are concentrations of lithologic types and primary bedding structures. Since only the upper portion of the valley train is exposed, the discussion of the depositional history is restricted to this portion. As shown in Figure 6, the upper part of the Mackinaw Member can be subdivided into a series of five deposits that are internally related. The earliest exposed deposit is clean quartz sand with a few scattered well-rounded pebbles. The dominant sedimentary structure is uniform planar cross-bedding in which foresets dip approximately 35 degrees. The surface of this sand body is extensively scoured, and gravel and sand deposits with prominent scour and fill cross-bedding rest on the eroded surface. Three lithologic types, coal, black shale, and rounded mudballs armored with small pebbles are very abundant. The coal and black shale give these deposits a distinctive dark color. The third deposit is sand and gravel which is largely flat-bedded and has only minor planar cross-bedding. These sands and gravels are devoid of coal and black shale clasts and have only a few small armored mudballs in them. Poorly bedded deposits of silt, clay, fine sand, and very small pebbles locally rich in organic matter overlie the flat-bedded sand and gravel. This is typical stream alluvium deposited by overbank flooding. Broad shallow channels filled with cobbles and small boulders lie in the alluvium. The clasts are generally well rounded and contain all lithologic types found in the valley train. Coal, black shale, and armored mudballs are particularly notable lithologic types. During

periods of high ground water levels a water table was established on the underlying alluvium so that the cobble-boulder gravel was saturated. During these periods calcium carbonate was deposited as cement to form a local conglomerate. The last water laid deposits in the valley train are alluvium similar to the alluvium beneath the channel fills. In areas outside the channels, the two deposits of alluvium are separated by a soil zone.

The earliest deposit at the head of the valley train is interpreted as outwash deposited during the advances of the early Woodfordian glaciers. The textures and planar cross-bedding of these deposits indicate transport by water with moderate current velocities. These velocities were great enough to produce cross-bedding, but were insufficient to cause erosion. The overlying gravels and sands with scour and fill cross-bedding appear to be closely related to the Woodfordian end moraines on the uplands shown in Figure 4. The crossing of the Illinois River valley by these moraines blocked the valley and ponded glacial meltwaters into a large deep lake (Wanless, 1957). Because of the depth of the water, only fine silt and clay were deposited on the lake floor. Finally the lake overflowed the dam, and the morainal material was swept away downstream. The outrushing water eroded the surface of the earlier outwash, producing an irregular surface. Bedrock was stripped from the walls of the Pekin-Sankoty Channel, and coal and black shale were deposited in the headward portion of the train. As the lake drained, mud on the lake floor was stripped away in blocks which were rolled down stream and picked up coatings of pebbles. Water velocities were so great at this time that deposits with scour and fill cross-bedding were laid down on top of each other and clasts as large as boulders greater than one meter in length were transported. As the flood waters waned, flow velocities decreased and flat-bed structures were produced. When the lake was completely drained, the surface of the valley train was above water level. The accumulation of floodplain alluvium appears to mark a period of fluctuating water levels in the Illinois River. It seems reasonable to assume that the fluctuations in the river level represent the advances and retreats of the Woodfordian glaciers after deposition of the moraines that formed the lake. After the period of floodplain deposition, a great flood poured through the Pekin-Sankoty Channel. This flood is attributed to great melting of the glaciers in late Woodfordian time, the Kankakee Torrent (Wanless, 1957). The narrows of the Pekin-Sankoty Channel constricted the meltwaters and formed a bottleneck that produced an outflow of great velocity and depth. This flood of water eroded much of the proximal end of the valley train, again eroded the bedrock walls of the Pekin-Sankoty Channel, and stripped more of the mud deposits from the old lake floor. Water velocity was so great that all material finer than cobbles was transported down stream leaving only the larger material as a lag deposit. Below the head of the valley train the flood waters carved the successive terraces on the surface of the valley train discussed above and illustrated in Figure 4. Finally, when the Kankakee flood had waned, the surface of the valley fill was covered with water only during periods of flooding of the Illinois River.

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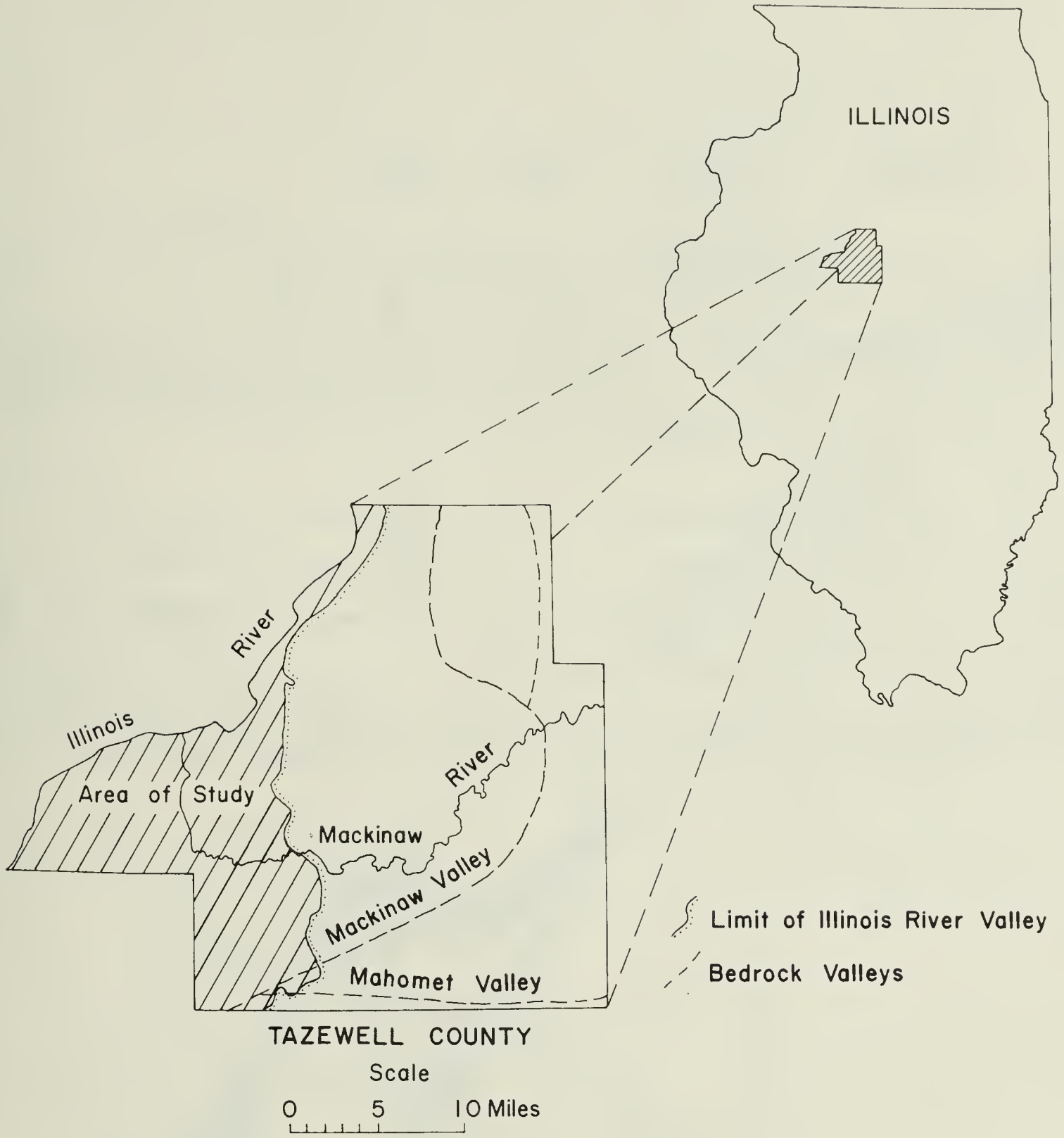

Fig. 1. Location map showing area of study and axis of Mackinaw and Mahomet Bedrock valleys.

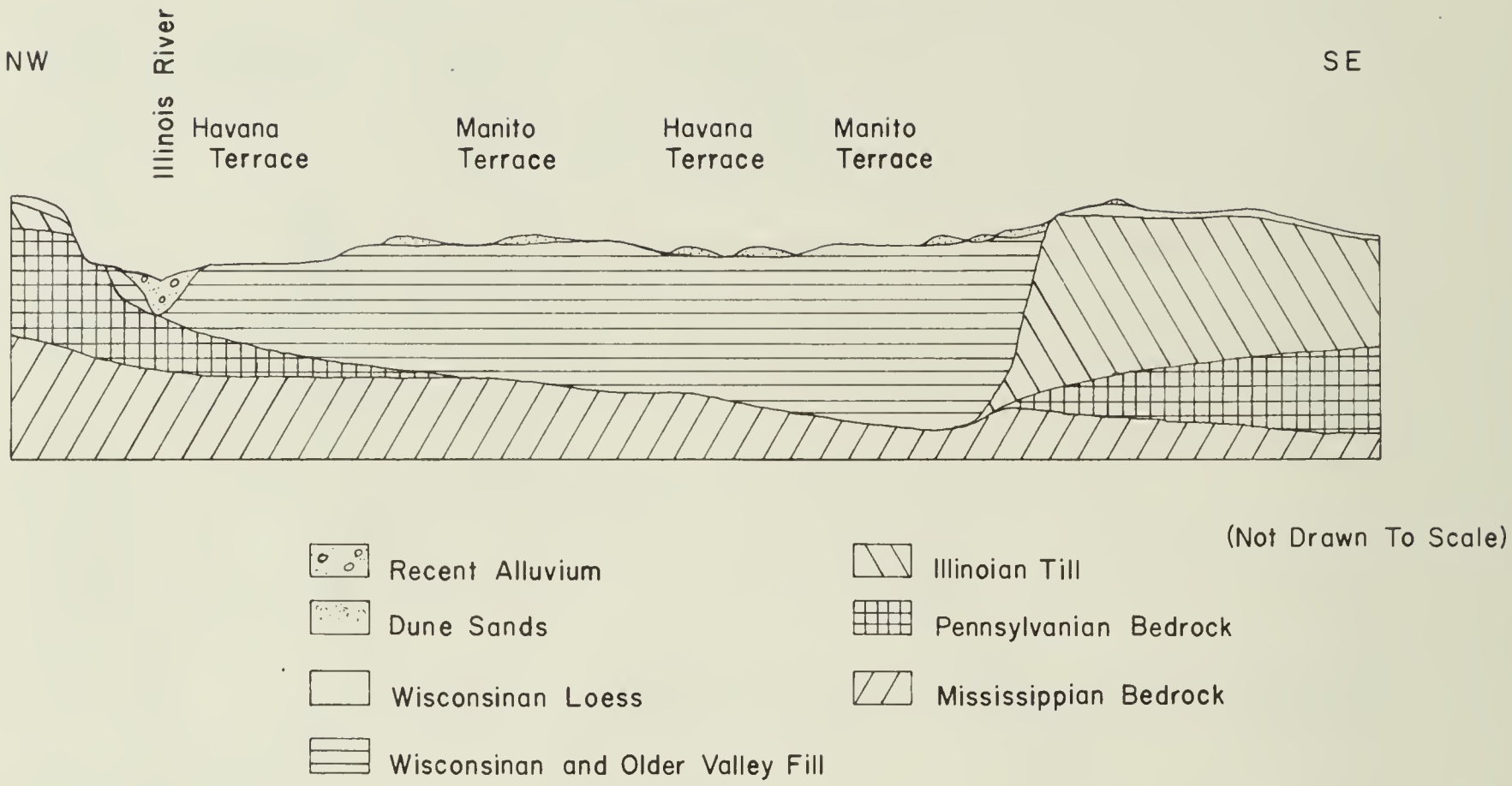

Fig. 2. Bedrock topography and bedrock exposures in Tazewell and Mason Counties.

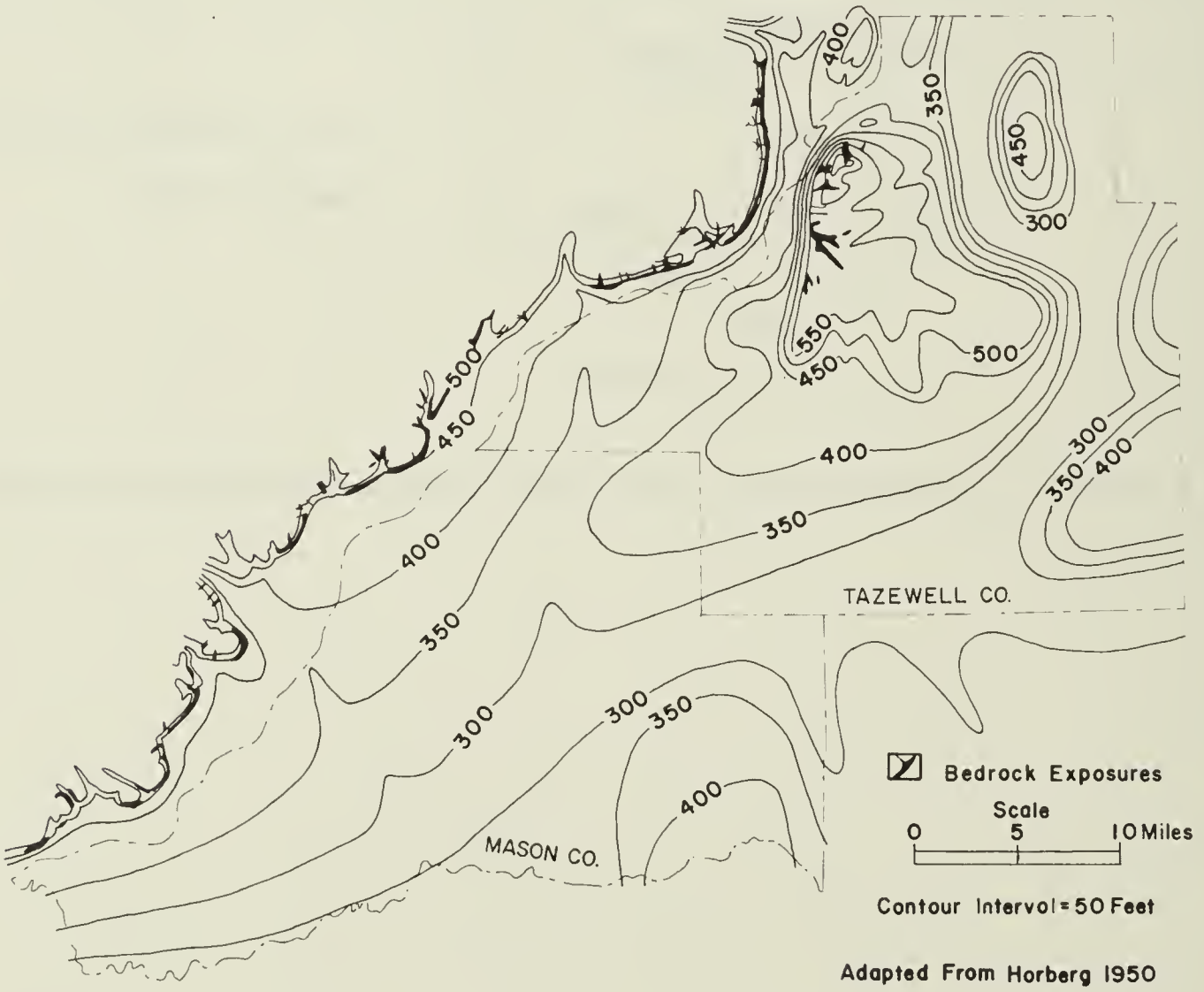

Fig. 3. Generalized cross section of the Illinois River valley in southern Tazewell County.

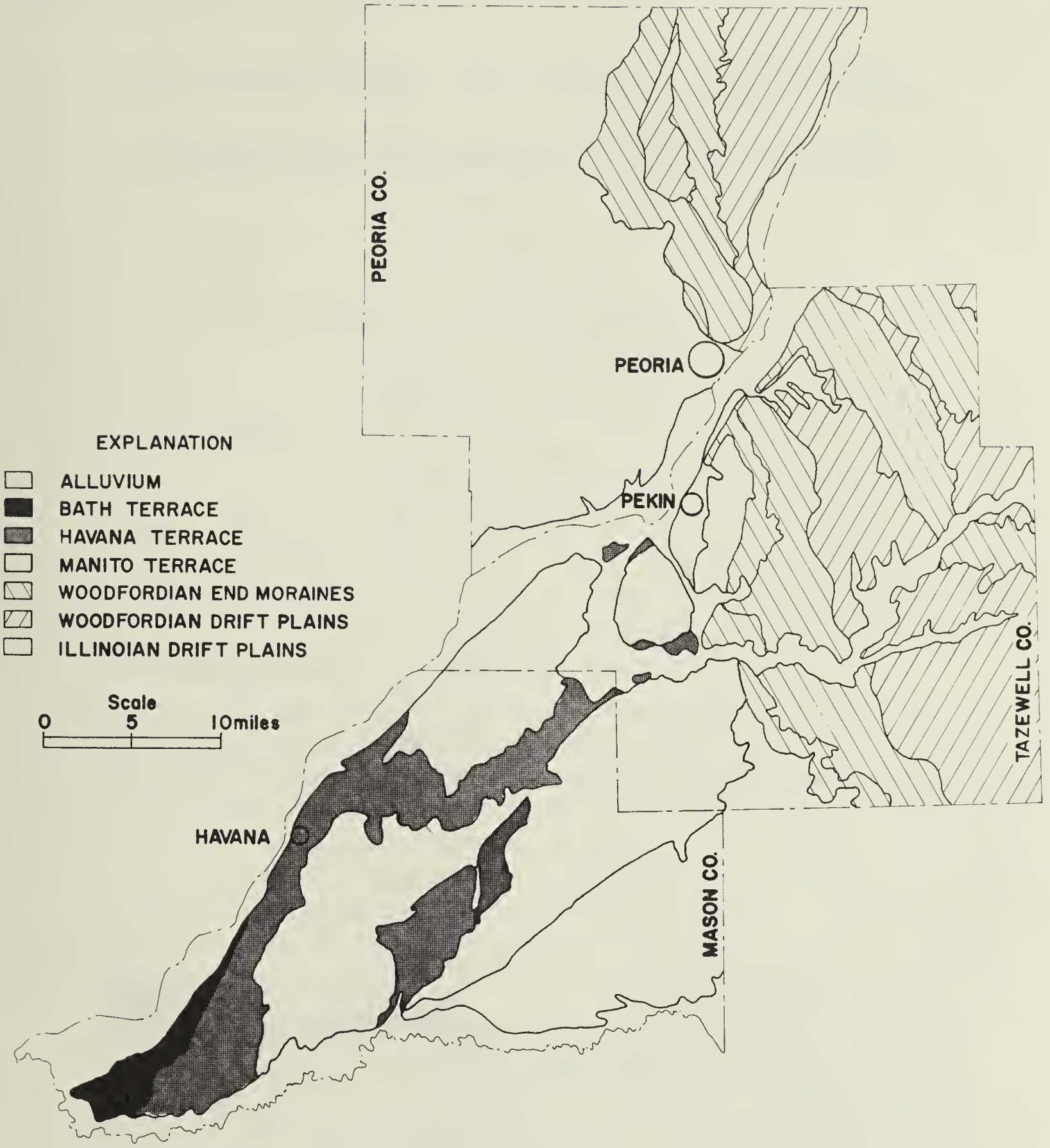

Fig. 4. Generalized surficial geologic map of Peoria, Tazewell, and Mason Counties.

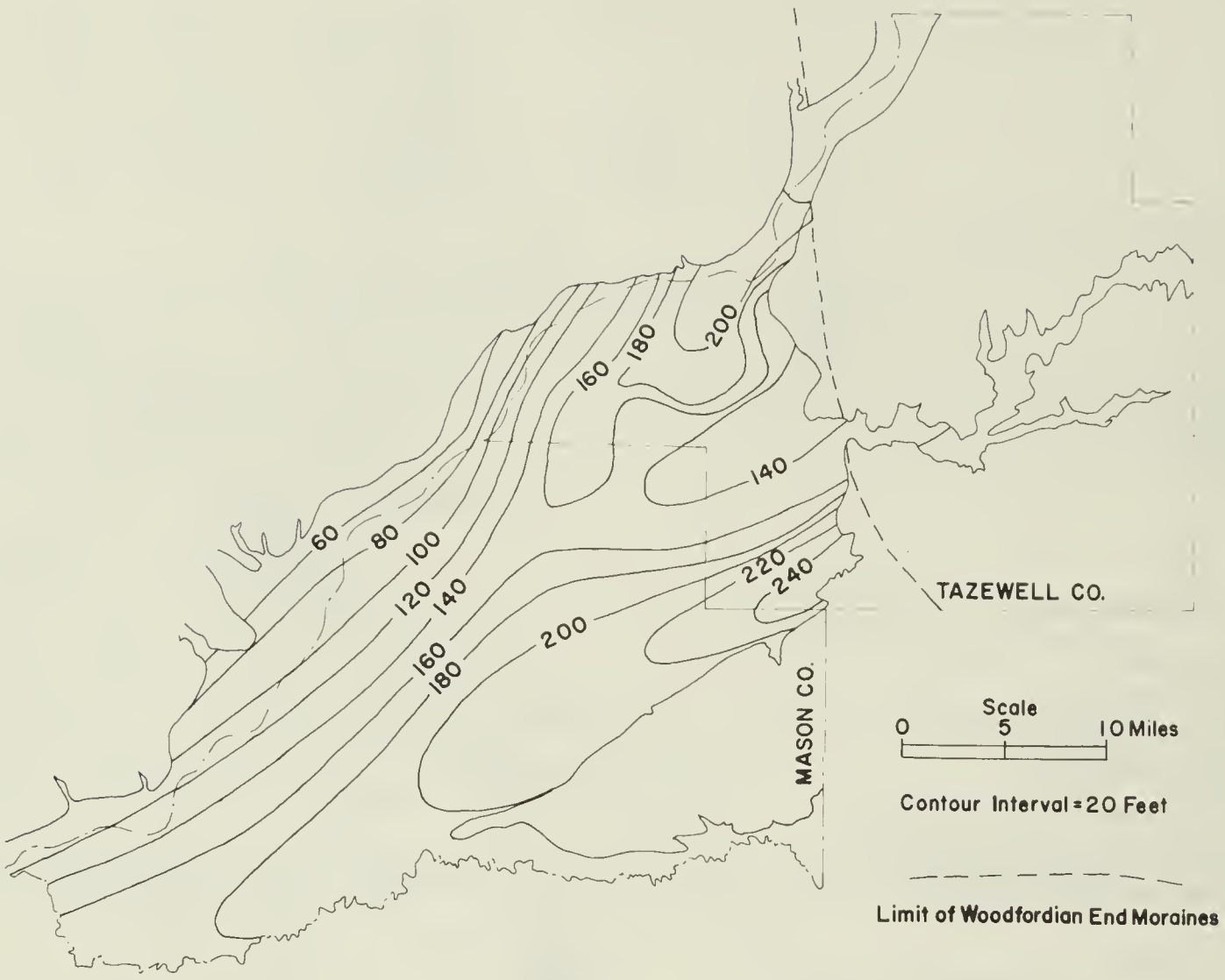

Fig. 5. Maximum thickness of fill in the Illinois River valley in Tazewell and Mason Counties.

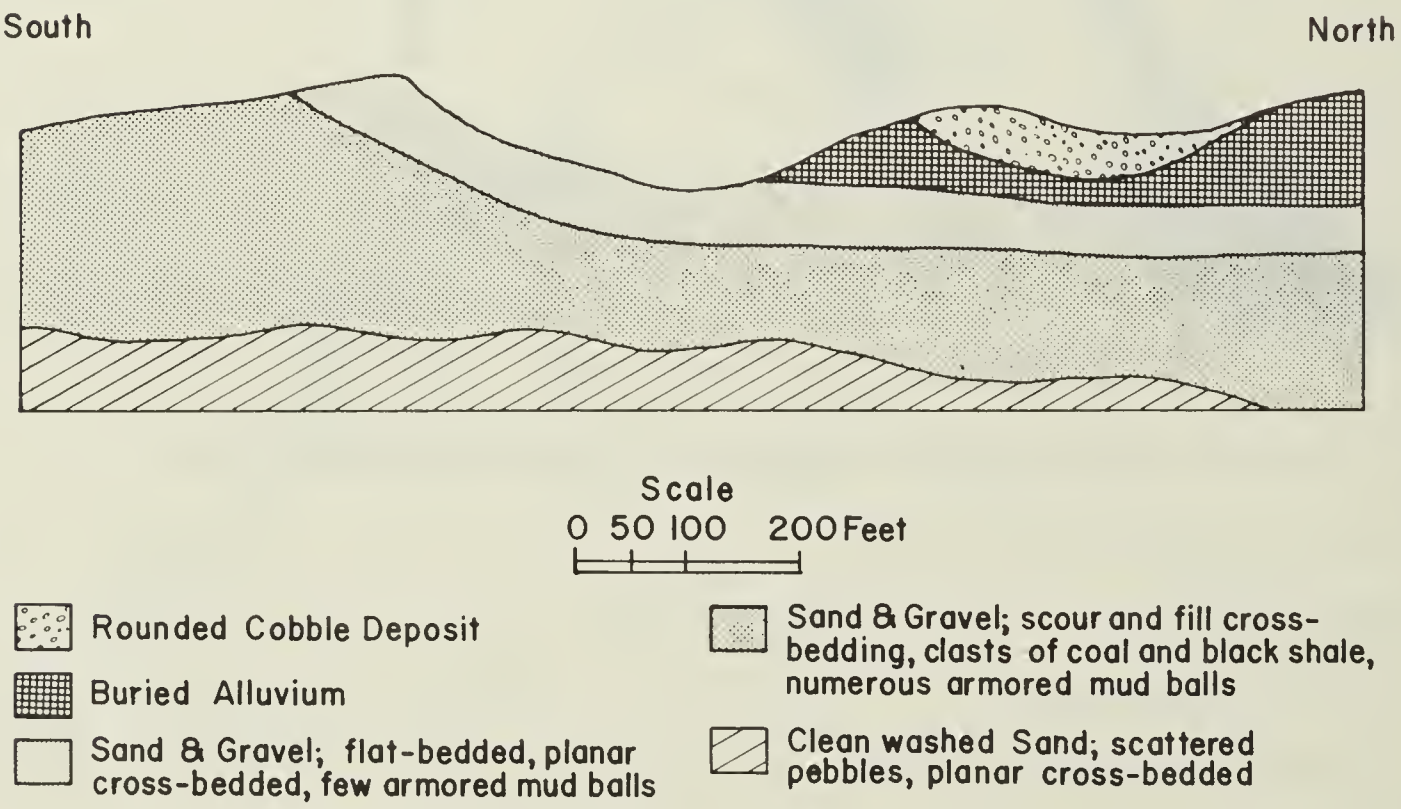
South
North
Scale
0 50 100 200 Feet
Rounded Cobble Deposit
Buried Alluvium
Sand & Gravel; flat-bedded, planar cross-bedded, few armored mud balls
Sand & Gravel; scour and fill cross-bedding, clasts of coal and black shale, numerous armored mud balls
Clean washed Sand; scattered pebbles, planar cross-bedded

Fig. 6. Diagrammatic cross section of Woodfordian deposits in the Illinois River valley south of Pekin.

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Vascular Flora of a Deep Coal Mine Refuse Reclamation Site in Illinois

J.R. Rastorfer[1],
Department of Biological Sciences,
Chicago State University
Chicago, IL 60628
and
G. Wilhelm[2],
The Morton Arboretum,
Lisle, IL 60532

ABSTRACT

The portions of the site that consisted of abandoned gob and slurry refuse were graded, amended, and seeded during reclamation efforts that were carried out in 1976 and 1977. A post-reclamation vascular plant inventory included the reclaimed areas and other segments of the site that were not directly subjected to the reclamation operations. In addition to the planted species, other floristic elements became established on the improved refuse materials. The flora consisted of about 250 taxa of vascular plants, of which approximately 30 species are considered new county records. Six principal growth forms were represented and consisted of 37 trees, 15 shrubs, 10 woody vines, 52 graminoids, 131 forbs, and 2 ferns.

INTRODUCTION

Overview

Prior to enactment of legislation governing coal mining operations, refuse materials from deep coal mines were generally disposed of above-ground. This was done with little or no attention paid to what might already have occupied the ground on which the materials were deposited. Coarse refuse (gob) was usually dumped adjacent to coal preparation facilities, where it accumulated into huge, steep-sided piles. Effluent materials (slurry) from coal washing facilities were pumped into impoundments near the preparation plants. These impoundments then became slurry ponds in which coal fines and other solids formed thick sediments several meters deep. After the mines were closed, the gob piles and slurry ponds were abandoned. Large areas of unaltered deep-mine refuse therefore re-

[1]Faculty Research Participant: Land Reclamation Program, Argonne National Laboratory, Argonne, IL 60439.
[2]Present Address: Department of Botany, Southern Illinois University, Carbondale, IL 62901.

mained on the landscape. There was little or no concern for the potentially adverse effects on both abiotic and biotic aspects of the environment (Martin, 1974; Zellmer and Carter, 1977).

Abandoned gob and slurry refuse areas in Illinois probably exceed 1800 hectares (ha) collectively. These land surfaces are aesthetically unpleasing and often are used locally as garbage dumps; furthermore, these areas have little real-estate or other potential economic value. Their presence may in fact depress local property assessments. In addition, they have caused numerous adverse ecologic effects on their environments. The refuse materials may become highly acidic and thus unfavorable for the establishment of vegetation. Without the protection of plant cover, consequently, the refuse materials are easily eroded, resulting in sediment outwash into adjacent land areas. Erosion also exposes fresh pyritic rock that is subject to oxidation resulting in acid runoff that adversely affects terrestrial and aquatic communities; furthermore, water with high concentrations of sulfates and heavy metals may enter ground-water systems (Nawrot et al., 1977; Zellmer, 1978; Zellmer and Carter, 1977).

In 1976, the Land Reclamation Program at Argonne National Laboratory, the Illinois Abandoned Mined Lands Reclamation Council, and the Illinois Institute of Natural Resources (now the Illinois Department of Energy and Natural Resources) began a cooperative project, the goals of which were to develop, demonstrate, and evaluate techniques for reclaiming abandoned gob and slurry refuse sites. Additional goals of the reclamation project were to (1) reduce the quantity of pollutants entering the surrounding landscape, (2) increase the economic potential of the affected and adjacent land, (3) improve the aesthetic value of the locality, and (4) assess several abiotic and biotic components (Zellmer, 1978; Zellmer and Carter, 1977; Zellmer and Wilkey, 1979).

Selection and Pre-Reclamation Description of the Site

The site selected was the abandoned Consolidated Coal Company Mine No. 14, which is located in Macoupin County, Illinois, near the village of Staunton (Figure 1). The mine was opened in 1904 and operated until the early 1920s. The coal seam (Herrin No. 6) was mined at approximately 85 m below the surface. The work force of about 500 men extracted up to 4550 metric tons of high-sulfur (5%) coal per day. The site has a total area of 13.8 ha, of which 9.3 ha had been affected directly by the mining operations and required reclamation (Zellmer and Carter, 1977).

The entire site consisted of three distinct parcels (Figures 2, 3A, 4A, and 5A): (Bernard, 1980; Zellmer, 1978 and 1979). The northern parcel featured, near its northern boundary, a dam built across a deep ravine. This dam impounded approximately 4.5 ha of water that was used for the mine's power plant, for the coal washing operations, and for pumping slurry from the coal washer. Most of the site, including the southern parcel, drained into this pond by means of a waterway through the middle parcel. Even after mining ceased, the pond continued to fill with sediments from the gob pile. These sediments consisted of coal fines and other solids that, along with the slurry sediments, accumulated up to 9 m (Figure 3A). After the dam failed in the early 1940s, rapid water runoff caused severe erosion, created gullies of 4 to 5 m in depth, and produced acid runoff and sediment outwash into the stream below the dam. From the time of the mine abandonment, the black deposits remained unvegetated except for the eventual presence of a few cattails (*Typha*) in one of the deep gullies; a few nonvascular plants, such as mosses and lichens, occurred on coal fines along some of the tree line margins. Less affected

segments of the northern parcel consisted of wooded areas, pasture lands, and cultivated fields (Figure 2).

The middle parcel, bounded by roads on the north and south, consisted mostly of about 1 ha of weedy pasture with a small stand of trees (Figure 4A). As mentioned previously, a waterway running through this parcel linked the southern and northern parcels. The adverse effects on vegetation by acid runoff were apparent along this waterway. Refuse sediment from the gob pile accumulated at the northern boundary of this parcel because of water impoundment caused by the culvert beneath Bunker Hill Road. This parcel had also become one of the site's major garbage dumps.

The southern parcel was characterized by a huge gob pile that reached an elevation of about 25 m above the surrounding landscape and covered nearly 1.8 ha (Figure 5A). During the 50 years following the closure of the mining operations, erosion cut deep gullies in the face of this refuse heap. No vegetation grew either on the gob pile or adjacent to it in those areas that had been subjected to severe acid runoff and sediment outwash. Within these affected areas, on the other hand, there were a few small patches of vegetation that escaped destruction because they were 30 to 60 cm above the outwash areas. Power plant and coal cleaning structures were located on the west side of the gob pile; however, only the foundations and the smokestack (55 m high) of these facilities remained. Most of the rails were removed from the railroad spur that had served the mine site; nevertheless, the entire right-of-way was still evident along the west and southwest boundaries of the parcel. Several stands of secondary-growth trees occupied small areas. The southern panhandle portion was disturbed by the mining operations, but apparently was not subjected to acid runoff from the eroded gob pile.

Pre-Reclamation Studies

Before the reclamation efforts began, the site was examined and sampled to assess several environmental factors: (1) quality of ground- and surface-water, (2) physical and chemical properties of the refuse materials and adjacent soils, (3) wildlife populations, (4) microbial populations in refuse materials and adjacent soils, and (5) some biotic aspects of the aquatic ecosystem. In addition, laboratory studies were made to investigate the effects of different soil amendments on the growth of selected plant species (Miller and Cameron, 1978; Schubert, 1979; Zellmer, 1978 and 1979; Zellmer and Wilkey, 1979). A detailed survey of the vascular plants occurring on the site was not made prior to the reclamation efforts, but a preliminary survey of the bryophytes was made by the senior author during a brief visit to the site in 1976.

Concomitant with the pre-reclamation investigations, the State of Illinois Abandoned Mined Lands Reclamation Council was in the process of obtaining titles to the three parcels discussed previously (Figure 2). Purchases and title transfers from the Consolidated Coal Company to the State of Illinois were completed by late summer of 1976. Legal ownership by the State of Illinois allowed the awarding of contracts for subsequent reclamation work. Marle, Inc., of Springfield, Illinois, was awarded a contract for construction work, and the project was directed by Stanley Zellmer of Argonne's Land Reclamation Program (Zellmer, 1978 and 1979; Zellmer and Carter, 1977).

Reclamation Efforts

Reclamation work began in September 1976. This initially required the removal of the old power plant and coal cleaning facility foundations, and accumulated

junk from the site. A borrow pit was dug to provide a mixture of topsoil, subsoil, and glacial till, which was stockpiled for subsequent use. The gob pile was graded down to an average height of about 8 m (approximately one-third its original height). Grading of the old slurry area was also initiated during this period.

After the grading and recontouring work was completed (Figure 6), refuse-amendment procedures began with the application and incorporation of acid-neutralizing agents into the upper 15 cm of the recontoured refuse surfaces. Several experimental plots on top of the recontoured gob pile area were left untreated. A cover material from the stockpile was placed over the treated refuse to a depth of 30 cm. A seed bed was prepared, including the application of agricultural limestone and fertilizer (N, P, K). Seeds mixed for drill planting consisted of *Festuca arundinacea*, *Lotus corniculatus*, *Phalaris arundinacea*, *Secale cereale*, and *Trifolium repens*. Other reclamation efforts included the installation of culvert pipe and water flow control structures, the excavation of a 0.5-ha retention pond, the construction of a dam, and the erection of new fencing. All of the site development endeavors mentioned above were completed before May 1977 (Zellmer, 1978; Zellmer and Carter, 1977; Zellmer and Wilkey, 1979).

Post-Reclamation Studies on the Site

Evaluation projects were carried out subsequent to the initial reclamation efforts. Projects included studies that concerned ground- and surface-water quality, aquatic ecosystems, revegetation assessments, characterizations of soils, rates of erosion, populations of soil microorganisms, wildlife surveys, and economic benefits (Jastrow et al., 1981; Miller and May, 1981; Sobek and Sullivan, 1981; Vinikour, 1981; Zellmer, 1979). In addition, Bernard (1980) has reported an interesting assessment of the views of local residents concerning the reclamation efforts.

Objectives of This Investigation

One of the objectives of this study is to make a detailed inventory of the vascular plants inhabiting the site, including both affected and non-affected areas (Figures 2 and 6). In addition, the vascular floristic components are characterized in each of the four major survey units discussed in the next section. Although qualitative in nature, the floristic information in this study is important in the establishment of a data base that will be necessary in future years to evaluate floristic changes on reclaimed deep-mine refuse areas.

METHODS FOR THE VEGETATIONAL SURVEYS AND COLLECTIONS

Survey Areas and Collections

Figure 6 shows the four major vegetational units that were surveyed among the three parcels of the site. These units were designated as those areas (1) dominated by trees and shrubs; (2) representing abandoned pastures; (3) altered by the mining operations, but not reclaimed; and (4) affected by the mining operations and subsequently graded, treated, and seeded. The surveys also included the margins of the three ponds on the site. Incidental collections of 17 species were made during 22-25 August 1978. Subsequently, more intensive surveys and collections of vascular plants were made 20-23 June 1979, 22-27 September 1979, and 18-19 May 1980.

Nomenclature and Floristics

The scientific names for the vascular plants follow those of Mohlenbrock (1975).

Species reported as new county records represent those taxa not reported for Macoupin County by Mohlenbrock and Ladd (1978). Mounted voucher specimens for all of the taxa reported were deposited in the herbarium of the Morton Arboretum in Lisle, Illinois.

The taxa, including those representing county records, are cited in a checklist arranged alphabetically by genus under eight categories: trees, shrubs, woody vines, sedges, grasses, rushes, forbs, and ferns. In the following text, the term "graminoid" includes the sedges, grasses, and rushes because of their superficially similar appearance. The term "forb" refers to all nonwoody vascular plants, with the exception of the graminoids and the ferns.

Vegetational Assessments

The characterization of the vegetation for the four major vegetational units are necessarily qualitative in nature owing to the inventory objectives and limitations. In general, the different areas are described with respect to their most conspicuous and apparent vegetational components. The three ponds on the site (Figure 6) are treated individually following the discussions of the four major vegetational units.

Although the forbs and graminoids in the ground cover of woodlands can be considered important in the characterization of plant communities, they have not been considered in detail here because these wooded areas have been greatly disturbed by grazing and other activities. A few noteworthy nonwoody species were found, nevertheless, in some isolated habitats within these areas and are mentioned.

OBSERVATIONS

Woodlands

The tree/shrub stands (Figure 6) within each of the three parcels of the site differed in character and composition because their recent histories were different; thus, they are discussed separately.

The areas dominated by trees and shrubs in the northern parcel were not affected extensively by the mining operations, but apparently were subjected to livestock grazing, at least periodically, before reclamation began. In addition, the perimetrical shapes of most of these stands were altered during the construction work. This can be seen clearly by comparing Figures 2 and 6. These woodland segments consisted mostly of *Carya ovata*, *Carya tomentosa*, *Fraxinus americana*, *Prunus serotina*, *Quercus imbricaria*, and *Quercus velutina* as the major tree taxa, along with *Celtis occidentalis*, *Diospyros virginiana*, *Sassafras albidum*, and other species of *Quercus*. Conspicuous shrubs included *Corylus americana*, *Lonicera maackii*, *Ribes missouriense*, *Rhus aromatica*, and *Rubus pensylvanicus*. A few vines worthy of mention included *Parthenocissus quinquefolia*, *Toxicodendron radicans*, *Rubus flagellaris*, and *Lonicera japonica*.

The middle parcel had only a small strip of woody vegetation. This was dominated by relatively young trees and shrubs, which apparently became established after mining ceased (Figure 6). The trees in this stand included *Juniperus virginiana*, *Prunus serotina*, *Quercus imbricaria*, *Quercus stellata*, *Robinia pseudoacacia*, *Sassafras albidum*, and *Ulmus americana*. Shrubs included *Lonicera maackii*, *Rhus glabra*, *Rubus pensylvanicus*, and *Sambucus canadensis*. Two vines were noted, i.e., *Lonicera japonica* and *Rubus flagellaris*.

The stands dominated by trees and shrubs in the southern parcel had very degraded understories, probably because of damaging effects from mining and

subsequent activities. Moreover, the trees in this parcel were generally younger than those in the northern and middle parcels. This was true especially along the abandoned railroad right-of-way in the southern parcel. The boundaries of the wooded areas in the southern parcel also were altered during the recontouring work (Figures 2 and 6). These wooded areas were different enough to warrant separate considerations.

The tree/shrub area located along the northwestern boundary of this parcel contained a mixture of trees consisting of *Acer saccharinum*, *Prunus serotina*, *Quercus imbricaria*, *Quercus velutina*, *Robinia pseudoacacia*, and *Sassafras albidum*. Shrubs were represented by *Ribes odoratum*, *Rosa multiflora*, *Rubus allegheniensis*, *Rubus pensylvanicus*, and *Sambucus canadensis*. Vines consisted of *Parthenocissus quinquefolia*, *Rubus flagellaris*, and *Toxicodendron radicans*.

The small circular-shaped tree stand about midway along the west side of the recovered gob pile area consisted of a single tree species, *Robinia pseudoacacia*. This group of trees was essentially without an understory.

The more-or-less oval-shaped stand of trees located in the upper portion of the panhandle was populated mostly with *Ulmus americana*, accompanied by *Celtis occidentalis*, *Crataegus crus-galli*, *Fraxinus pennsylvanica* var. *subintegerrima*, *Prunus serotina*, *Quercus imbricaria*, *Salix amygdaloides*, and *Salix interior*.

The long narrow wooded area along the abandoned railroad right-of-way consisted mostly of scattered seedlings of *Acer saccharinum*, *Crataegus* spp., *Populus deltoides*, and *Robinia pseudoacacia*. Shrubs in this area included *Rhus glabra* and *Sambucus canadensis*.

Abandoned Pastures

Two abandoned pasture segments occurred in the northern parcel (Figure 6). These areas and the adjacent wooded stands were actively pastured until 1976. They have remained abandoned since the site development began, except for periodic invasions by cattle and goats. These pasture segments were essentially the same with respect to species composition. The most abundant plants were *Festuca arundinacea*, *Poa pratensis*, and *Tridens flavus*, with *Asclepias syriaca* and *Rosa multiflora* also present.

Areas Affected by Mining but Not Reclaimed

A highly disturbed strip of land (Figure 6) occurred along the western boundary of the southern parcel, and for the most part corresponded to a portion of the abandoned railroad right-of-way. It was not part of the reclamation effort because of property easement disagreements between the state and the village of Staunton. The western margin of this area included a tree row (not shown in Figure 6) that was composed mostly of *Celtis occidentalis*, *Maclura pomifera*, and *Populus deltoides* intermixed with *Quercus* spp. The graminoids of the affected area consisted of *Carex annectens*, *Panicum virgatum*, *Poa pratensis*, *Sorghastrum nutans*, and *Tridens flavus*. Forbs included *Achillea millefolium*, *Apocynum sibiricum*, *Chenopodium album*, *Helianthus annuus*, *Lotus corniculatus*, *Rumex crispus*, *Trifolium pratense*, *Verbascum thapsus*, and *Vernonia missurica*.

Areas Recontoured, Treated, and Seeded

The largest portions of the site represent those areas of the three parcels that were greatly altered by the mining operations, abandoned for about 50 years, and then recently subjected to intensive reclamation tasks, during which these land surfaces were graded, edaphically amended, and seeded, all in similar fashion.

The subsequent development of vegetation in these three areas differed, however, and thus will be discussed separately below.

The rectified land of the northern parcel had a conspicuously dense coverage of graminoids (Figures 3B and 6) in which the major components were *Festuca arundinacea*, *Phalaris arundinacea*, and *Tridens flavus*, with lesser amounts of *Bromus commutatus*, *Bromus tectorum*, *Hordeum pusillum*, *Juncus tenuis*, and *Poa pratensis*. Numerous forbs were scattered throughout the area, including *Ambrosia artemisiifolia*, *Geranium carolinianum*, *Geum canadense*, *Lotus corniculatus*, *Oxalis dillenii*, *Polygonum lapathifolium*, *Polygonum pensylvanicum* var. *laevigatum*, *Potentilla simplex*, *Pycnanthemum tenuifolium*, *Rumex acetosella*, *Solanum carolinense*, *Specularia perfoliata*, *Trifolium repens*, and *Veronica peregrina*. Invading trees were *Populus deltoides* and *Salix interior*.

The reclaimed land in the middle parcel (Figures 4B and 6) was also characterized by graminoids consisting of *Agrostis alba*, *Festuca arundinacea*, *Dactylis glomerata*, *Phalaris arundinacea*, and *Tridens flavus*. Associated forbs included *Ambrosia trifida*, *Chenopodium album*, *Eupatorium serotinum*, *Lactuca serriola*, *Lotus corniculatus*, *Plantago aristata*, *Plantago lanceolata*, and *Solidago canadensis*.

The constructed land in the southern parcel had a varied distribution of herbaceous vascular plants, especially in the area occupied by the graded gob pile. This area was quite varied topographically and was characterized by a flat-topped hill (the regraded gob pile) that had relatively steep slopes down to level land (Figures 5B and 6). Graminoid components of the vegetation included *Agrostis alba*, *Bromus commutatus*, *Bromus inermis*, *Dactylis glomerata*, *Festuca arundinacea*, *Hordeum pusillum*, *Juncus interior*, *Panicum lanuginosum* var. *implicatum*, *Panicum virgatum*, *Phalaris arundinacea*, *Poa chapmaniana*, *Poa pratensis*, *Schizachyrium scoparium*, and *Setaria viridis*. Numerous forbs were also found in this area, namely, *Ambrosia trifida*, *Arenaria serpyllifolia*, *Aster pilosus*, *Cassia fasciculata*, *Cerastium* sp., *Croton capitatus*, *Erigeron annuus*, *Lepidium densiflorum*, *Lepidium virginicum*, *Lotus corniculatus*, *Melilotus* spp., *Myosotis virginica*, *Plantago virginica*, *Ranunculus abortivus*, *Sibara virginica*, *Silene antirrhina*, *Solidago gigantea*, *Veronica arvensis*, *Veronica peregrina*, *Viola rafinesquii*, and *Xanthium strumarium*.

Pond Margins

There are three ponds on the site, two in the northern parcel, and one in the middle parcel (Figure 6). The floristic elements, if any, associated with these ponds, are discussed below.

The small pond in the northeastern portion of the northern parcel was established prior to the initial site development efforts. Conspicuous marginal species were *Eleocharis obtusa*, *Erechtites hieracifolia*, and *Leersia oryzoides; Lemna minor* was a common floating species.

Typha latifolia was the most abundant marginal plant around the pond in the central portion of the northern parcel; however, *Juncus effusus* var. *solutus* also occurred here, and *Phragmites australis* had invaded the northern tip of the pond just below the dam.

The margin of the small pond at the southern boundary of the middle parcel was essentially devoid of vegetation, undoubtedly because of its generally persistent acid conditions.

DISCUSSION

Species Composition Compared to Known Vascular Flora for Macoupin County

Mohlenbrock and Ladd (1978) report 934 species of tracheophytes for Macoupin County; the present list of 247 species thus makes up about 25% of the county's known vascular plant flora. This number of species is appreciable in view of the relatively small area of the reclaimed site and the site's recent history. The list also contains 32 species that, according to Mohlenbrock and Ladd (1978), have been heretofore unknown from Macoupin County. The taxa in this category can be regarded as county records and are marked by an asterisk (*) in the checklist.

Interesting and Noteworthy Species

The trees *Amelanchier arborea* and *Cercis canadensis*, the forbs *Arisaema triphyllum* and *Trillium recurvatum*, and the ferns *Asplenium platyneuron* and *Botrychium virginianum* were confined to the slopes of the woodland in the northwestern portion of the northern parcel. These plants and the local prevalence of species of *Quercus* and *Carya* indicate that the site probably represents a remnant oak-hickory community.

Many specimens of *Quercus velutina* were atypical, their leaves and buds suggesting that hybridization may have occurred with *Quercus rubra*. Also, numerous individuals of *Ulmus americana* were somewhat atypical, particularly with respect to leaf size and general growth habit. The atypical forms were especially apparent in the woodlot in the upper panhandle of the southern parcel. The irregular morphological features of the elm trees in this location may have been caused, at least in part, by the adverse environmental conditions associated with the mining operations. Ashby and his co-workers, on the other hand, have shown that many species of trees can grow normally on some unamended minesoils (Ashby, 1964; Ashby et al. 1978).

Concluding Remarks

As mentioned previously, the originally exposed gob and slurry sediments (pre-reclaimed surface materials) of the site were essentially devoid of vegetation (Figures 3A, 4A, and 5A). The few cattails in a gully in the slurry sediment area of the northern parcel were exceptions. On the other hand, Nawrot et al. (1977) report the occurrence elsewhere in Illinois of numerous plant taxa on some abandoned deep-mine refuse lands consisting of gob, slurry sediments, and tipple waste. Certainly, seeds of numerous forbs, graminoids, and trees from adjacent areas of the site (Figure 2) were readily available to the pre-reclaimed substrata. Among the different environmental parameters, however, high temperatures were probably as effective as any other factor in preventing the establishment of seedlings (Deely and Borden, 1973; Schramm, 1966). High acidity, poor water retention capacity, and other edaphic conditions also may have been critical factors in preventing the establishment of plants on the abandoned gob material.

In contrast, the reworked and edaphically treated minesoil of the site is currently supporting vegetation (Figures 3B, 4B, and 5B). In addition, most of the plant taxa now present apparently were not in the seed mixture used on the treated minesoils, but arrived as disseminules from surrounding areas. Besides forbs and graminoids, these formerly unvegetated areas support invading trees such as *Populus deltoides*, *Robinia pseudoacacia*, and *Salix* spp.

In addition to the aesthetic benefits that the new vegetational cover has brought

to the site, other environmental improvements are apparently developing. The grasses and forbs, which are currently the dominant plants on the constructed minesoil, provide additional food and shelter for wildlife. Furthermore, this vegetational cover, along with the previously established tree stands, will very likely enhance habitat conditions for wildlife, because now there is a much greater plant species diversity than existed on the site prior to the initial reclamation effort (Holland, 1973; Zellmer, 1979).

Edaphic factors such as fertility and organic content undoubtedly will increase with each succeeding growing season. The legumes, such as *Desmanthus*, *Desmodium*, *Lespedeza*, *Lotus*, *Medicago*, *Melilotus*, and *Trifolium* are particularly important because they bear nitrogen-fixing bacteria. The prevalence of graminoids should contribute considerably to an accumulation of litter. This organic matter should foster greater populations of heterotrophic microorganisms that play a significant role in decomposition and nutritent cycling (Miller and May, 1981).

The establishment of a vegetational cover stabilizes the soil surface and thus is instrumental in reducing erosion. A reduction in soil erosion in turn improves surface water quality by reducing acidity and sediment outwash. Eventually, the aquatic communities of the site will benefit from better surface water conditions (Vinikour, 1981).

It is apparent that many of the earlier adverse environmental aspects of the site have been rectified as a result of the reclamation recovery effort; however, many processes in edaphic and biotic development require long periods of time. The complete reclamation of the site, therefore, cannot be realized until the planned objectives have been achieved.

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CHECKLIST OF VASCULAR PLANTS[1]

TREES

Acer (Aceraceae)
- *A. negundo* L.
- *A. saccharinum* L.

Amelanchier (Rosaceae)
- *A. arborea* (Michx. f.) Fern.

Carya (Juglandaceae)
- *C. ovata* (Mill.) K. Koch
- *C. tomentosa* (Poir.) Nutt.

Celtis (Ulmaceae)
- *C. occidentalis* L.

Cercis (Leguminosae)
- *C. canadensis* L.

Corylus (Betulaceae)
- *C. americana* Walt.

Crataegus (Rosaceae)
- *C. crus-galli* L.
- *C. pruinosa* (Wendl.) K. Koch

Diospyros (Ebenaceae)
- *D. virginiana* L.

Fraxinus (Oleaceae)
- *F. americana* L.
- *F. pennsylvanica* var. *subintegerrima* (Vahl) Fern.

Gleditsia (Leguminosae)
- *G. triacanthos* L.

Juniperus (Cupressaceae)
- *J. virginiana* L.

Maclura (Moraceae)
- *M. pomifera* (Raf.) Schneider

Morus (Moraceae)
- **M. alba* L.
- *M. rubra* L.

Populus (Salicaceae)
- *P. deltoides* Marsh.

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[1]Asterisk (*) indicates new county record.

Prunus (Rosaceae)
P. serotina Ehrh.

Quercus (Fagaceae)
Q. alba L.
Q. imbricaria Michx.
Q. marilandica Muenchh.
Q. muhlenbergii Engelm.
Q. rubra L.
Q. stellata Wangh.
Q. velutina Lam.

Robinia (Leguminosae)
R. pseudoacacia L.

Salix (Salicaceae)
S. amygdaloides Anderss.
S. discolor Muhl.
**S. fragilis* L.
S. interior Rowlee
S. nigra Marsh.
**S. rigida* Muhl.

Sassafras (Lauraceae)
S. albidum (Nutt.) Nees

Ulmus (Ulmaceae)
U. americana L.
U. rubra Muhl.

SHRUBS

Berberis (Berberidaceae)
**B. thunbergii* DC.

Cornus (Cornaceae)
C. racemosa Lam.

Lonicera (Caprifoliaceae)
**L. maackii* Maxim.

Malus (Rosaceae)
M. ioensis (Wood) Britt.

Rhus (Anacardiaceae)
R. aromatica Ait.
R. glabra L.

Ribes (Saxifragaceae)
**R. ordoratum* Wendl.
R. missouriense Nutt.

Rosa (Rosaceae)
R. carolina L.
**R. multiflora* Thunb.
R. setigera Michx.

Rubus (Rosaceae)
R. allegheniensis Porter
R. occidentalis L.
R. pensylvanicus Poir.

Sambucus (Caprifoliaceae)
S. canadensis L.

WOODY VINES

Campsis (Bignoniaceae)
C. radicans (L.) Seem.

Cocculus (Menispermaceae)
**C. carolinus* (L.) DC.

Lonicera (Caprifoliaceae)
**L. japonica* Thunb.

Parthenocissus (Vitaceae)
P. quinquefolia (L.) Planch.
**P. vitacea* (Knerr) Hitchc.

Rubus (Rosaceae)
R. flagellaris Willd.

Smilax (Smilacaceae)
S. hispida Muhl.

Toxicodendron (Anacardiaceae)
T. radicans (L.) Kuntze

Vitis (Vitaceae)
V. cinerea Engelm.
V. riparia Michx.

SEDGES (Cyperaceae)

Carex
- **C. annectens* Bickn.
- *C. artitecta* Mack.
- *C. blanda* Dewey
- *C. brevior* (Dewey) Mack.
- *C. bushii* Mack.
- **C. cephalophora* Muhl.
- *C. cristatella* Britt.
- **C. muhlenbergii* var. *enervis* Boott
- **C. umbellata* Schk.

Cyperus
- **C. ferruginescens* Boeckl.

Eleocharis
- *E. erythropoda* Steud.
- **E. obtusa* (Willd.) Schult.
- *E. obtusa* var. *detonsa* (Gray) Drap. & Mohlenbr.

GRASSES (Gramineae)

Agrostis
- *A. alba* L.

Alopecurus
- *A. carolinianus* Walt.

Andropogon
- *A. virginicus* L.

Bromus
- *B. commutatus* Schrad.
- *B. inermis* Leyss.
- *B. tectorum* L.

Danthonia
- *D. spicata* (L.) Beauv.

Dactylis
- *D. glomerata* L.

Echinochloa
- *E. pungens* (Poir.) Rydb.

Elymus
- *E. canadensis* L.
- *E. hystrix* L.
- *E. villosus* Muhl.
- *E. virginicus* L.

Festuca
- *F. arundinacea* Schreb.

Hordeum
- *H. jubatum* L.
- *H. pusillum* Nutt.

Leersia
- *L. oryzoides* (L.) Swartz

Panicum
- *P. dichotomiflorum* Michx.
- *P. lanuginosum* var. *implicatum* (Scribn.) Fern.
- *P. virgatum* L.

Phalaris
- **P. arundinacea* L.

Phleum
- *P. pratense* L.

Phragmites
- **P. australis* Trin.

Poa
- *P. chapmaniana* Scribn.
- *P. compressa* L.
- *P. pratensis* L.

Schizachyrium
- *S. scoparium* (Michx.) Nash.

Secale
- **S. cereale* L.

Setaria
- *S. lutescens* (Weigel) Hubb.
- *S. viridis* (L.) Beauv.

Sorghastrum
- *S. nutans* (L.) Nash

Sphenopholis
- *S. obtusata* var. *major* (Torr.) Erdman

Tridens
- *T. flavus* (L.) Hitchcock

Tripsacum
- *T. dactyloides* L.

Vulpia
- *V. octoflora* (Walt.) Rydb.

RUSHES (Juncaceae)

Junus
J. brachycarpus Engelm.
**J. effusus* var. *solutus* Fern. & Wieg
J. interior Wieg
J. tenuis Willd.

FORBS

Achillea (Compositae)
A. millefolium L.

Agrimonia (Rosaceae)
A. pubescens Wallr.

Allium (Liliaceae)
A. canadense L.

Amaranthus (Amaranthaceae)
**A. ambigens* Standl.

Ambrosia (Compositae)
A. artemisiifolia L.
A. trifida L.

Antennaria (Compositae)
A. plantaginifolia (L.) Richards.

Apocynum (Apocynaceae)
A. sibiricum Jacq.

Arctium (Compositae)
A. minus (Hill) Bernh.

Arenaria (Caryophyllaceae)
**A. serpyllifolia* L.

Arisaema (Araceae)
A. triphyllum (L.) Schott

Asclepias (Asclepiadaceae)
A. hirtella (Pennell) Woodson
A. incarnata L.
A. syriaca L.

Aster (Compositae)
A. novae-angliae L.
A. pilosus Willd.

Barbarea (Cruciferae)
**B. vulgaris* var. *arcuata* (Opiz) Fries

Bidens (Compositae)
B. aristosa L.
B. aristosa var. *retrorsa* (Sherff) Wunderlin
B. comosa (Gray) Wieg.

Capsella (Cruciferae)
C. bursa-pastoris (L.) Medic.

Cardamine (Cruciferae)
C. pensylvanica Muhl.

Carduus (Compositae)
**C. nutans* L.

Cassia (Leguminosae)
C. fasciculata Michx.

Cerastium (Caryophyllaceae)
C. sp.
**C. vulgatum* L.

Chaerophyllum (Umbelliferae)
C. procumbens (L.) Crantz

Chamaesyce (Euphorbiaceae)
C. maculata (L.) Small.

Chenopodium (Chenopodiaceae)
C. album L.

Cichorium (Compositae)
C. intybus L.

Cirsium (Compositae)
C. vulgare (Savi) Tenore

Claytonia (Portulacaceae)
C. virginica L.

Commelina (Commelinaceae)
C. communis L.

Croton (Euphorbiaceae)
C. capitatus Michx.

Desmanthus (Leguminosae)
D. illinoensis (Michx.) MacM.

Desmodium (Leguminosae)
D. dillenii Darl.

Ellisia (Hydrophyllaceae)
E. nyctelea L.

Erechtites (Compositae)
E. hieracifolia (L.) Raf.

FORBS (Cont'd)

Erigeron (Compositae)
E. annuus (L.) Pers.
E. canadensis L.
E. philadelphicus L.

Erythronium (Liliaceae)
E. albidum Nutt.

Eupatorium (Compositae)
E. altissimum L.
E. rugosum Houtt.
E. serotinum Michx.

Fragaria (Rosaceae)
F. virginiana Duchesne

Galium (Rubiaceae)
G. aparine L.
G. circaezans Michx.

Geranium (Geraniaceae)
G. carolinianum L.

Geum (Rosaceae)
G. canadense Jacq.

Helianthus (Compositae)
H. annuus L.
H. grosseserratus Martens
H. strumosus L.

Hieracium (Compositae)
**H. gronovii* L.

Holosteum (Caryophyllaceae)
H. umbellatum L.

Hypericum (Hypericaceae)
H. punctatum Lam.

Ipomoea (Convolvulaceae)
I. hederacea (L.) Jacq.

Lactuca (Compositae)
L. canadensis L.
L. serriola L.

Lamium (Labiatae)
**L. amplexicaule* L.

Lemna (Lemnaceae)
L. minor L.

Lepidium (Cruciferae)
L. campestre (L.) R. Br.
**L. densiflorum* Schrad.
L. virginicum L.

Lespedeza (Leguminosae)
L. virginica (L.) Britt.

Liatris (Compositae)
L. aspera Michx.

Lippia (Verbenaceae)
L. lanceolata Michx.

Lotus (Leguminosae)
**L. corniculatus* L.

Medicago (Leguminosae)
M. sativa L.

Melilotus (Leguminosae)
M. alba Desr.
M. officinalis (L.) Lam.

Monarda (Labiatae)
M. bradburiana Beck.

Myosotis (Boraginaceae)
M. virginica (L.) BSP.

Myosurus (Ranunculaceae)
M. minimus L.

Ornithogalum (Liliaceae)
**O. umbellatum* L.

Osmorhiza (Umbelliferae)
O. longistylis var. *villicaulis* Fern.

Oxalis (Oxalidaceae)
O. dillenii Jacq.
O. violacea L.

Parietaria (Urticaceae)
P. pensylvanica Muhl.

Pastinaca (Umbelliferae)
P. sativa L.

Penstemon (Scrophulariaceae)
P. digitalis Nutt.
P. pallidus Small

Phytolacca (Phytolaccaceae)
P. americana L.

Plantago (Plantaginaceae)
P. aristata Michx.
P. lanceolata L.
P. rugelii Dcne.
P. virginica L.

Podophyllum (Berberidaceae)
P. peltatum L.

FORBS (Cont'd)

Polygala (Polygalaceae)
P. sanguinea L.

Polygonatum (Liliaceae)
P. commutatum (Schult.) A. Dietr.

Polygonum (Polygonaceae)
P. convolvulus L.
P. lapathifolium L.
P. pensylvanicum var. *laevigatum* Fern.
P. punctatum Ell.
P. virginianum L.

Potentilla (Rosaceae)
P. simplex Michx.

Pycnanthemum (Labiatae)
P. tenuifolium Schrad.

Ranunculus (Ranunculaceae)
R. abortivus L.

Rorippa (Cruciferae)
**R. islandica* var. *fernaldiana* Butt. & Abbe.

Rumex (Polygonaceae)
R. acetosella L.
R. crispus L.

Sanicula (Umbelliferae)
S. canadensis L.

Sibara (Cruciferae)
S. virginica (L.) Rollins

Sida (Malvaceae)
S. spinosa L.

Silene (Caryophyllaceae)
S. antirrhina L.

Smilacina (Liliaceae)
S. racemosa (L.) Desf.

Solanum (Solanaceae)
S. carolinense L.

Solidago (Compositae)
S. canadensis L.
S. gigantea Ait.
S. nemoralis Ait.
S. missouriensis Nutt.
S. speciosa Nutt.

Specularia (Campanulaceae)
S. perfoliata (L.) A. DC.

Staphylea (Staphyleaceae)
S. trifolia L.

Taraxacum (Compositae)
T. officinale Weber

Thlaspi (Cruciferae)
T. arvense L.

Tradescantia (Commelinaceae)
T. ohiensis Raf.

Tragopogon (Compositae)
**T. dubius* Scop.

Trifolium (Leguminosae)
T. hybridum L.
T. pratense L.
T. repens L.

Trillium (Liliaceae)
T. recurvatum Beck

Typha (Typhaceae)
T. latifolia L.

Valerianella (Valerianaceae)
V. radiata (L.) Dufr.

Verbascum (Scrophulariaceae)
V. thapsus L.

Vernonia (Compositae)
V. missurica Raf.

Veronica (Scrophulariaceae)
V. arvensis L.
V. peregrina L.

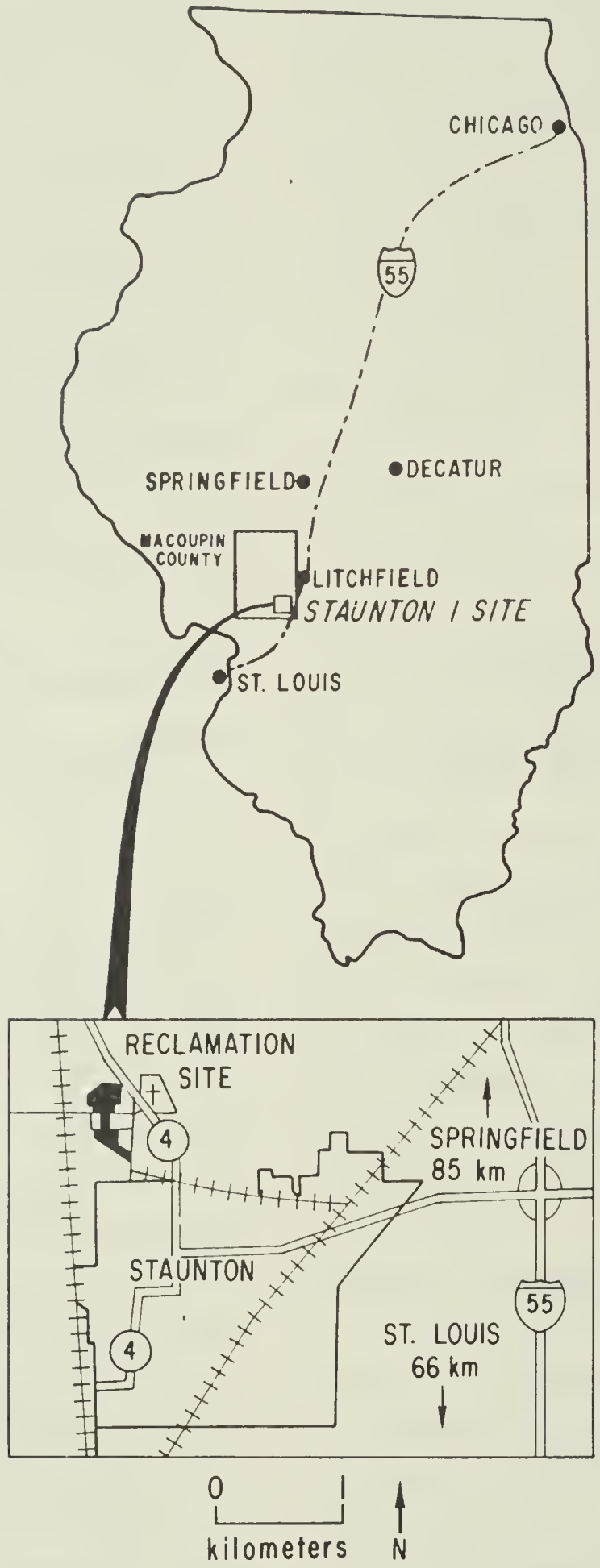
Viola (Violaceae)
V. rafinesquii Greene
V. sororia Willd.

Xanthium (Compositae)
X. strumarium L.

FERNS

Asplenium (Polypodiaceae)
A. platyneuron (L.) Oakes

Botrychium (Ophioglossaceae)
B. virginianum (L.) Sw.


CHICAGO
55
SPRINGFIELD
DECATUR
MACOUPIN COUNTY
LITCHFIELD
STAUNTON I SITE
ST. LOUIS
RECLAMATION SITE
4
SPRINGFIELD
85 km
STAUNTON
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55
ST. LOUIS
66 km
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kilometers
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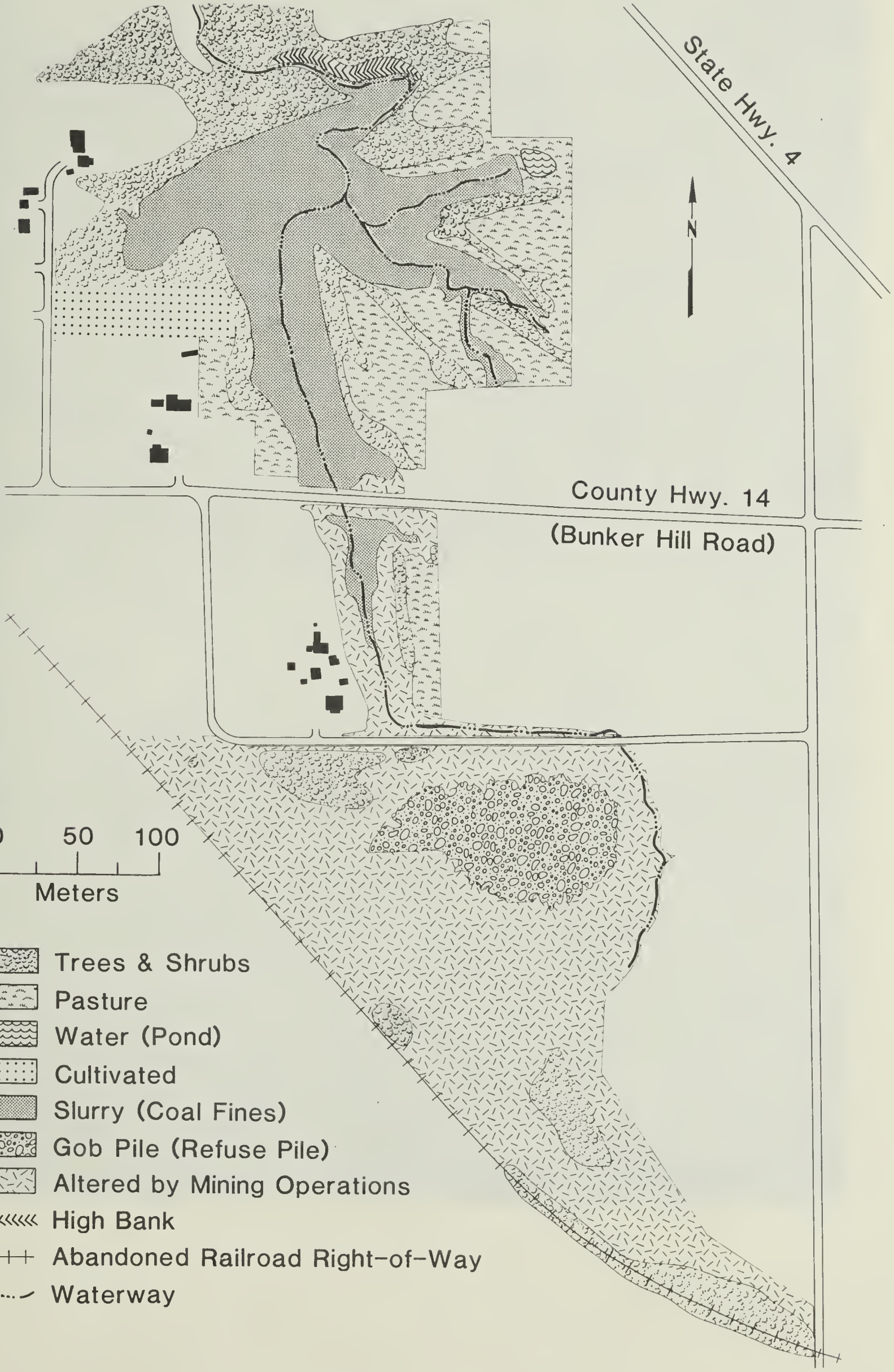
PRE-RECLAMATION



A

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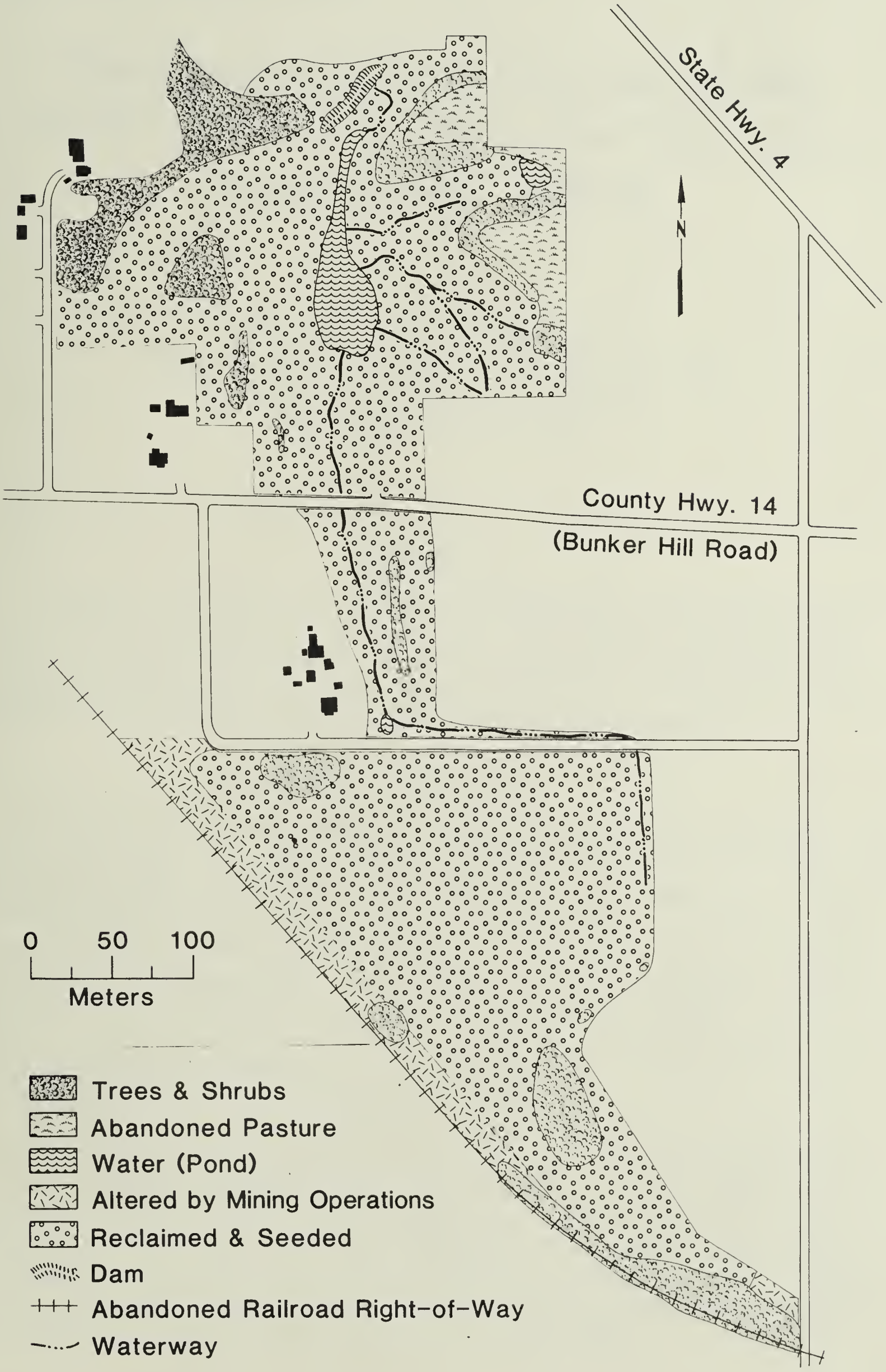
POST-RECLAMATION



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Photodocumentation of the Loss of Hill Prairie Within Pere Marquette State Park, Jersey County, Illinois

William E. McClain
Illinois Department of Conservation
Division of Forest Resources and Natural Heritage
Lincoln Towers, 524 South 2nd Street
Springfield, Illinois 62706

ABSTRACT

Comparisons of 1937 and 1974 aerial photographs indicate that five hill prairies within Pere Marquette State Park have been reduced in size by 61.9 percent due to woody plant invasion. This study confirms the reports of other investigators and indicates that many of the hill prairies in Illinois will disappear within the next 20 to 25 years unless prescribed burning is implemented.

INTRODUCTION

In Illinois, hill or bluff prairies are prominent features of the landscape along most of the major river systems. Although these hill prairies were often large and conspicuous, they were not mentioned in the early scientific literature, or mapped by the Government Land Office Surveyors during the 1820's and 1830's. Worthen, a geologist with the Illinois Geological Survey, was the first to describe these grass-covered bluffs (Worthen 1868). Since the initial description by Worthen, numerous authors have studied the hill prairies in various geographic regions of the state, but the publication of Evers (1955), who studied hill prairies of the Illinois and Mississippi Rivers and compiled annotated listings of their vegetation, is the most comprehensive paper on the hill prairies in Illinois.

In Jersey County, Illinois, loess hill prairies are prominent on many of the south-facing ridges along the Illinois and Mississippi Rivers. Although hill prairies were mentioned by Hamilton (1919), the first scientific studies of these prairies were not completed until the 1960's (Kilburn and Ford 1963, Kilburn and Warren 1963, Bland and Kilburn 1966, Ranft and Kilburn 1969). These studies provided considerable information on the soils, vegetation, and environment of the hill prairies near Elsah in Jersey County. Their research also indicated that many of the hill prairies were being invaded by woody vegetation.

For many years, ecologists have observed the gradual deterioration of hill prairies due to woody plant invasion. This phenomenon has been described by Kilburn and Warren (1963), Reeves, Zimmerman and Ebinger (1978), and Ebinger (1981), but no data are available on the extent of the invasion or the amount of time required for a hill prairie to be overgrown with woody plants. Consequently, a study was initiated to determine the extent of woody invasion on five hill prairie remnants within Pere Marquette State Park using 1937 and 1974 aerial photography.

DESCRIPTION OF THE STUDY AREA

The five hill prairies that were studied are located in Township 6N, Range 13W, Sections 9 and 10, Brussels Quadrangle, U.S.G.S. 7.5 minute series. This portion of the park lies within the Driftless Section of the Middle Mississippi Border Division (Schwegman 1973). This geographic region is characterized by steep topography and numerous outcrops of dolomite, limestone, and shale, particularly within Pere Marquette State Park. The most widespread and exposed geologic formation in the area is the Pleistocene loess which forms a thick mantle which varies in

The soil of the hill prairies is Hamburg silt which is essentially unaltered loess (Fehrenbacher and Downey 1966). This soil is limey throughout and grass-covered rather than forested like most of the soils within Pere Marquette State Park. The upper horizon of these soils is very friable and grayish-brown to brown in color (Fehrenbacher and Downey 1966).

With the exception of the hill prairies, the ravines and ridges within Pere Marquette State Park are forested. An examination of the Government Land Office Survey Records indicates that the area was forested during presettlement times. Since the time of settlement in the 1820's and 1830's, the forests have been used as a source of lumber and fuel.

Due to its exceptional scenic features and the interests of local citizens, land was purchased in 1932 for the purposes of establishing a state park. Fire was excluded from the park until 1973 when it was introduced by Department of Conservation personnel in their prairie management program.

METHODS

The peripheries of five hill prairies within Pere Marquette State Park were defined on 1937 (Scale = 1: 19,385) and 1974 (Scale = 1: 24,375) black and white aerial photographs. The peripheries as defined on these photographs were measured to the nearest one-hundreth of an acre using a Lasico Series 40 Electronic Planimeter. The results were tabulated and comparisons were made with the 1937 and 1974 data to determine the extent of woody invasion.

RESULTS AND DISCUSSION

Comparisons of the 1937 and 1974 acreages for the five hill prairies indicate that they have been greatly reduced in size due to woody plant invasion (Table 1). With the exception of the First Lookout Prairie, all others show a reduction in size with the prairie at Twin Mounds most reduced (Table 1). When the totals of all five prairies are combined, the figures indicate that the prairies have been reduced in size by 61.9 percent in the 37 year period from 1937 to 1974.

The primary invaders on these prairies are gray dogwood (*Cornus drummondii*), smooth sumac (*Rhus glabra*), and flowering dogwood (*Cornus florida*). Despite the more xeric nature of the prairie as compared to the forest, woody plants readily establish themselves (Reeves, Zimmerman and Ebinger 1978).

Ebinger (1981) in the study of glacial drift hill prairies in east-central Illinois, regarded the prairies as "transient communities" that are "commonly completely overgrown with woody vegetation". Kilburn and Warren (1963) concluded that hill prairies, in the absence of fire, will be permanently converted to forest. This study of hill prairie remnants within Pere Marquette State Park supports these conclusions and indicates that the entire acreages of small hill prairies or significant portions of large hill prairies can be permanently converted to forest in 35 years or less. The rate of decline of the prairies is dependent upon slope, soil moisture, and size of the prairie. Under present climatic conditions and without the use of prescription burning, it seems likely that a great majority of the hill prairies in Illinois will be lost to woody invasion within the next 20 to 25 years unless vigorous management plans are devised and implemented.

ACKNOWLEDGEMENTS

Special appreciation is extended to Dr. Peter J. Roberts for determining the acreages of the hill prairies on the 1937 and 1974 aerial photographs.

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Table 1. A comparison of 1937 and 1974 acreages for five hill prairies within Pere Marquette State Park.

	Size In Acres	
Name of Prairie	1937	1974
Goat Cliff	2.00	1.00
McAdams Peak	2.51	0.90
Twin Mounds	14.00	2.42
Twin Shelters	5.00	3.46
First Lookout	1.50	2.00
Totals	25.01	9.78

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Nuclear Dry Mass and Area of Free and Crowded Human Buccal Cells Observed in Smears

G.A. Pappelis, T. Kupcek, and A.J. Pappelis
President's Scholar; Research Aide; and,
Professor, Department of Botany,
Southern Illinois University,
Carbondale, Illinois 62901, respectively.

INTRODUCTION

Cellular and nuclear dry mass and shape changes have been related to human buccal cell development, maturation, senescence, and death (1, 3, 5, 8). Parabasal cells (Pb) and their nuclei increase in dry mass and size as they enlarge to form intermediate cells with round nuclei (I-R). These degenerate to form cells in the following seqence: intermediate cells with oval nuclei (I-O); intermediate cells with rod-pyknotic nuclei (I-RP); cells with pyknotic nuclei (P); and, anucleate cells. Typical averages for nuclear dry mass in these cell types are as follow (3): Pb, 43 pg; I-R, 69 pg; I-O, 51 pg; I-RP, 48 pg; and P, 35 pg. Typical averages for nuclear areas in these cell types are as follow (3): Pb, 70×10^{-8} cm^2; I-R, 92×10^{-8} cm^2; I-O, 86×10^{-8} cm^2; I-RP, 51×10^{-8} cm^2; and P, 23×10^{-8} cm^2. Similar trends were reported for cells of the oral mucosa of the upper lip. These studies formed the basis of the Cell Development Index proposed for use in the study of oral exfoliation studies (5).

Bhattacharya *et al.* (1) used the Cell Development Index to select cells in buccal smears for study of eight nuclear traits: total nucleic acids, DNA, RNA, total protein, histone, non-histone protein, protein-bound lysine, and protein-bound arginine. All of these nuclear indices (except total nucleic acid and RNA) increased significantly as Pb cells became I-R cells. All of the nuclear indices decreased drastically as I-R cells degenerated through the sequence I-O, I-RP, and P. Their data supported the sequence of cellular and nuclear changes described in the Cell Development Index.

One problem was common to both quantitative interference microscopy (3, 5, 6, 7, 8) and quantitative cytofluorescence microscopy (1) when buccal cells were studied in smears: finding sufficient number of flat, free cells for measurement. This is especially difficult when both cellular and nuclear traits are to be studied. Members of this laboratory working with two types of true interference micro-

scopes, Namarski interference microscopy, and phase contrast-epifluorescence microscopy have observed all of the cell types described for oral cells in smears (5) and variations like those reported in vaginal and cervical smears (4, 9, 10). In addition to free buccal cells, many have folded or curled margins or are exfoliated in sheets or clumps. Many are damaged during collection or in preparation of smears. It would be advantageous to use any cell within a type whether free (with or without folds, curls, and overlap) or in sheets and clumps if the object to be studied is clearly visible (especially the nuclei). These traits have been used as cytological aids to monitor hormone levels in the blood that influences vaginal and cervical cell development, maturation, senescence, and exfoliation (9, 10) using phase contrast microscopy to identify cells. The comments by Hammond (2) regarding the naming and use of these indices (Folded- and Crowded-Cell Indices) in gynecologic cytology is not our concern in this study. Since we can identify these variations in cells in buccal smears with interference microscopy, we thought that some concern should be given to them.

The purpose of this study was to compare the nuclear dry mass (NDM) and nuclear area (NA) means for I-R cells in smears from a male donor showing the following traits: free cells that are flat compared to free cells that have folded or curled margins; and, free cells that are flat compared to cells in sheets or clumps. We also observed the cells from male and female donors for the above traits and the presence or absence of nucleoli in cell types used in the Cell Development Index.

MATERIAL AND METHODS

Buccal cells were obtained by gently drawing a finger across the mucosa to the corner of the lips. These cells were transferred by touching the finger to a glass slide. The cells were suspended in one drop of distilled water and covered with a cover glass which was then sealed to the slide with paraffin oil. NDM and NA of I-R cells were determined using a Leitz interference microscope as described in earlier studies of buccal cells (3, 5, 6, 7, 8). The same donor was used throughout (male, 10 years old). One smear per day was prepared for study. The Lietz interference microscope (Horn; Mach-Zender) was equipped with 50x A 0.85/∞/0.17 objectives and 16x Periplan GF occulars (field diameter 0.3 mm, 800x total magnification) and Orthomat camera (10x ocular). Both fringe and even field modes were used with polychromatic or monochromatic (546 nm) light to study cells and their inclusions. The quantitative determinations of optical path difference (between cytoplasm and nucleus) was made with the even field mode and monochromatic light. Nuclear area was determined by projecting negatives, tracing outlines, and planimetry of tracings (compared to stage micrometer negatives projected at the same distance).

Two quantitative studies were conducted. In the first, NDM and NA of I-R cells were compared using five flat (without folded or curled margins) cells and five cells in sheets or clumps (more than three cells in each) per slide in each of six slides (replications). In the second, NDM and NA of I-R cells were compared using five flat cells and five cells with either folded or curled margins per slide in each of six slides (replications). All cells were free of others except as described (sheets, clumps). Cells in sheets and clumps have been defined as crowded cells (9, 10).

Nucleolar observations were made in four buccal smears from males (ages 19 through 45) and four females (ages 18 through 28). Cells in these smears were ex-

amined to determine whether intermediate cells existed in the conditions described above (flat; folded or curled margins; free; in sheets or clumps of three or more cells).

RESULTS AND DISCUSSION

The grand means for the six replicates (30 cell means) for free cells (without folded or curled margins) and cells in sheets or clumps were: free cells, 69.2 pg and 92.1 $\times 10^{-8}$ cm^2; and, cells in sheets or clumps, 65.0 pg and 98.0 $\times 10^{-8}$ cm^2. The ranges for NDM and NA for the 30 free cells were: NDM, 44.1 to 118.0 pg; and, NA, 56.6 to 152.9 $\times 10^{-8}$ cm^2. The ranges for NDM and NA for the 30 cells in sheets or clumps were: NDM, 35.1 to 126.2 pg; and, NA, 62.2 to 134.7 $\times 10^{-8}$ cm^2. When the six replicate means (five cells of each type per replicate) for both NDM and NA were compared by analysis of variance, no significant differences were found between the two cell types (5% level).

The grand means for the six replicates (30 cell means) for free cells (without folded or curled margins) and free cells with either folded or curled margins were: free cells (no folds or curls), 69.8 pg and 98.3 $\times 10^{-8}$ cm^2; and, free cells with folded or curled margins, 70.5 pg and 103.0 $\times 10^{-8}$ cm^2. The ranges for NDM and NA for the 30 cells without folded or curled margins were: NDM, 44.2 to 90.3 pg; and, NA, 69.5 to 139.5 $\times 10^{-8}$ cm^2. The ranges for NDM and NA for 30 cells with folded or curled margins were: 46.8 to 108.7 pg; and, NA, 46.8 to 146.0 $\times 10^{-8}$ cm^2. When the six replicate means (five cells of each type per replicate) were compared using analysis of variance, no significant differences were found between the two cell types (5% level).

The results of the two studies described above were like those reported earlier (means and ranges) for I-R cells (3). Because there were no significant differences between flat, free cells, free cells with folded or curled margins, or cells in sheets or clumps, we conclude that any of these cell types are acceptable for study of these and/or other nuclear traits in I-R cells. We believe that this will also apply to cells of other types described in the Cell Development Index. The replicate means for NA and NDM for all treatments in the present study fall near the regression line presented by Pappelis *et al* (8). Similarly, the correlation coefficient using these means for NA and NDM was high ($r = 0.80$) and compares favorably with that reported earlier (8); $r = 0.93$.

The exfoliation pattern in vaginal tissue and the morphology of the cells is affected by blood hormone levels (4, 9, 10). Our earlier observations that both male and female buccal smears show cells of this type (used in folded and crowded cell indices) were confirmed in the present study of four male and four female donors. What this means in terms of hormonal influences on the oral mucosa is not known. Care must be taken in any interpretation of this data since these cytological indices have received criticism (2) as they were applied in gynecologic studies.

All of the cell types were observed in the four male and four female oral smears. All of the Pb cells from the eight donors contained nucleoli. The number of nucleoli in intermediate cells varied from donor to donor but their overall means were similar: male, 24% of all intermediate cells contained nuclei; and, female, 26% of all intermediate cells contained nucleoli. Nucleoli were not observed in P cells. We had no difficulty in differentiating between nucleoli and Barr bodies in cells of buccal smears from female donors. We believe that there is a difference in size of nucleoli in the cell types (nucleoli in Pb cells appear to be larger than those in in-

termediate cells) and that the size may be related to cell development, maturation, and senescence. This must be confirmed using specific nucleolar stains to varify that the objects called nucleoli in degenerating cells observed in this study with interference microscopy are not chromocenters.

ABSTRACT

The nuclear dry mass (NDM) and area (NA) means for free, flat intermediate cells with round nuclei (I-R) (NDM, 69.8 pg; NA, 98.3 $\times$ 10^{-8} cm^2) were not statistically different from those of flat I-R cells with folded or curled margins (NDM, 70.5 pg; NA, 103.0 $\times$ 10^{-8} cm^2). Similarly, the NDM and NA means for free, flat I-R cells (NDM, 69.2 pg; NA, 92.1 $\times$ 10^{-8} cm^2) were not statistically different from I-R cells exfoliated in sheets or clumps containing more than three cells (NDM, 65.0 pg; NA, 98 $\times$ 10^{-8} cm^2). We conclude that any I-R cells (and infer that any other cell types) found free with or without folds or curls in margins or in sheets or clumps in buccal smears can be used for nuclear studies (by cell type) related to buccal cell development, maturation, senescence, and death. All parabasal cells appear to contain nucleoli while only 25% of intermediate cells and none of the pyknotic cells contain nucleoli. It may be that nucleolar size and dry mass is an important trait to include in future studies of buccal cell development, maturation, and senescence.

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Methods of Determining Sex and Body Size in Prehistoric Samples of White-tailed Deer (*Odocoileus virginianus*)

James R. Purdue
Zoology Section
Illinois State Museum
Springfield, Illinois 62706

ABSTRACT

Modern samples of *Odocoileus virginianus* are used to determine (1) sex and (2) size of prehistoric conspecifics. A special combination of standardization of measurements and discriminant function analysis is proposed and evaluated. An example, distal ends of tibias from the Brown Site (23VE3), Vernon County, Missouri, is used to illustrate the procedures.

INTRODUCTION

Knowledge of the sex, biological age, and body size represented by bones of white-tailed deer (*Odocoileus virginianus*) recovered from prehistoric sites is important for reconstructions of paleoecology and aboriginal subsistence practices. Many bones from mature individuals can be distinguished from those of immature animals by the extent of epiphyseal fusion (Purdue, 1983). The determination of sex is not as easily evaluated since only the frontal and pelvic bones have qualitative differences (Edwards et al., 1982). White-tailed deer are sexually dimorphic in body size and, thus, the bones also differ in size. However, quantification of bone size is complicated by overlap between males and females and by clinal variation of deer from different geographic and temporal settings (e.g., Richie, 1970). Methods are presented in this paper that determine sex and estimate mean size of individuals in a sample of deer from a particular geographic and temporal setting. The practicability of the methods is demonstrated on a sample of zooarchaeological data.

EXAMPLE DATA

The transverse diameter (width) and anterior-posterior diameter (depth) of the distal ends of tibias were measured for samples of modern deer from Jo Daviess

County, Illinois, 15 counties in central Illinois, and the Savannah River Plant, Aiken and Barnwell counties, South Carolina. The modern samples of tibias were collected from deer either killed by vehicles or brought to hunter check-stations. An enzyme cleaner (Biz) was used to remove the soft tissue. Tibias from an archaeological context, the Brown Site (23VE3), Vernon County, Missouri, were also measured. The Brown Site was occupied by the Great Osage Division of the Osage Tribe from the late 1600's to about 1774 AD (Chapman, 1974). The tibias from all of the deer samples had ossifying or ossified epiphyseal sutures and, consequently, were of adult proportions (Purdue, 1983).

DETERMINATION OF SEX

Three options will be shown for determining sex as indicated by a set of measurements on a bone. All options are variations of two basic statistical techniques, standardization with z-scores and discriminant function analysis (DFA). The z-score (z),

$$z = \frac{x_i - \bar{x}}{s},$$

indicates the number of standard deviation units a measurement (x_i) is from the mean ($\bar{x}$) when $\bar{x}$ is set at 0.0 and the standard deviation (s) at 1.0.

The z-scores are entered as variables in DFA, a multivariate procedure which uses the reference specimens, e.g., the modern samples of deer, to develop criteria for determining the sex for individuals in a prehistoric sample (see Cooley and Lohnes, 1971, and Davis, 1973, for general discussions of DFA). DFA is also used to reclassify the individuals in the reference samples so that the degree of morphological overlap between sex classes can be determined.

Option 1. A normal statistical procedure for the determination of sex is to use the mean and standard deviation for the entire sample to standardize and enter the data into DFA. The flaw in this arrangement is that when there are systematic differences in body size between localities, as evidenced by white-tailed deer, then deleterious systematic trends in sex assignment by DFA are introduced (Table 1). Males from small-sized populations, e.g., South Carolina, and large females, e.g., Illinois, are poorly discriminated. Clinal variation is the basis of the problem because males from some localities are the size of females at others and the resulting z-scores are an uncomfortable mix of sex and clinal effects. The classification results are identical to those produced by no standardization.

Option 2. Another method is to independently analyze each sample. This involves computing a mean and standard deviation for each sample before standardizing and then performing DFA on each separate sample. The maximum amount of separation between the sexes is produced for each sample and systematic differences in body size between localities no longer are an issue (Table 1). Unfortunately, the prehistoric samples can no longer be analyzed for sex because there is no appropriate reference group.

Option 3. The final option is a hybrid of the first two. Each sample, using its respective mean and standard deviation, is standardized independently of the others. The males, which in each sample tend to have larger measurements, have

positive z-scores and the females, which are smaller, have negative values. The z-scores of all individuals are then analyzed simultaneously with DFA. The results indicate that the systematic problems apparent in Option 1 are resolved and that the sexes can be separated with near the efficiency of Option 2 (Table 1). The critical factor in the procedure is that both sexes are represented in each sample. Ideally, the sex ratio should be 1:1. Various degrees of departure from this ratio were investigated and, fortunately, the ratio can deviate considerably (approximately ± 30%) from the ideal and still produce satisfactory results (Fig. 1). Although not done here, an independent test of the sex ratio is to note the number of each sex represented by the pelvic and frontal bones. Unfortunately both elements tend to be fragmented in archaeological sites and the frontal, in particular, may be biased for males because of the presence of antler which was often used for tools. Overall, Option 3 appears to be the best method for determining sex and it will be utilized in the next section.

ESTIMATING MEAN SIZE OF AN UNKNOWN SAMPLE

Even using the optimal technique there is overlap in the size of the sexes of deer and DFA is unable to distinguish sex correctly all the time (Table 1). Results of DFA on the modern samples can be used to "correct" for the effects of the misclassifications in the unknown or zooarchaeological sample. Descriptive statistics, i.e., mean and standard deviation, are calculated for the known or modern samples, thus producing a set of actual values, i.e., values that estimate the parameters of the population with morphological overlap of the sexes. A set of apparent descriptive statistics is also calculated for the known samples by reassigning those individuals that DFA placed in the wrong sex. The apparent set of values is directly comparable with the descriptive statistics for the unknown sample sexed by DFA. Further, the means of the unknown sample can be altered for comparison with the actual means of known deer by the following correction:

$$\bar{x}_u = \bar{x}_a \left(\frac{1}{g} \sum_{1}^{g} \frac{\bar{r}_i}{\bar{a}_i} \right)$$

where $\bar{x}_u$ = the corrected mean of one sex for the unknown sample, $\bar{x}_a$ = the apparent mean of the same sex for the unknown sample, $\bar{r}_i$ = the actual mean of the same sex for the i'th known sample, $\bar{a}_i$ = the apparent mean of the same sex for the i'th known sample, and g = the number of known samples. The correction factor is the proportion of actual to apparent values averaged over the number of known samples.

There are several characteristics worthy of note in the example of the application of the method (Table 2). The differences between the actual and apparent values are slight, ranging from near 0 to approximately 2%. The apparent means for the males are slightly larger relative to the actual values and the reverse is true for the females. The apparent standard deviations for both sexes are smaller then the actual values. These effects are the result of the small males being classified by DFA as females and large females as males. Therefore, apparent sex groups are

more homogeneous resulting in lower variance measures. In general, the individuals that are reclassified by DFA are the small males and the large females that are close to the same size and the descriptive statistics generated from actual and apparent data approximate each other.

DISCUSSION

The special application of data standardization described here yields an estimate of the sex ratio of a sample of prehistoric deer. Under normal circumstances, the larger the unknown sample size, the greater is the probability that the critical factor, i.e., both sexes are present, is satisfied. However, because of the possible vagarities of the actual prehistoric sex ratio as well as the inherent overlap in size of the sexes, the sex ratio produced by these statistical procedures for a sample should be considered tentative. In contrast, the estimate of mean size is more robust since individuals misassigned by sex approximate each other in bone measurements and produce a compensating effect when descriptive statistics are calculated. The estimates of mean size can be used for further statistical procedures, such as projection of body weight by regression analysis and comparison to other samples by principal component analysis. While the methods presented here are specifically designed for application to white-tailed deer, they could obviously be applied to other species where sexual dimorphism is present.

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Table 1. Classification by sex of white-tailed deer (*Odocoileus virginianus*) from three localities using Discriminant Function Analysis (SPSS; MAHAL option; prior probabilities set equal to the proportions of males and females in the sample; Klecka, 1975) on 2 measurements on the distal end of the tibia.

		Percent Classified					
		Male			Female		
Location[a]	Option[b]	n	MasM[c]	MasF	n	FasF	FasM
JD	1	23	91.3	8.7	39	71.8	28.2
	2	23	78.3	21.7	39	89.7	10.3
	3	23	78.3	21.7	39	89.7	10.3
CI	1	14	85.7	14.3	20	70.0	30.0
	2	14	71.4	28.6	20	80.0	20.0
	3	14	57.1	42.7	20	90.0	10.0
SC	1	26	19.2	80.8	31	100.0	0.0
	2	26	65.4	34.6	31	93.5	6.5
	3	26	73.1	26.9	31	87.1	12.9

[a]JD = Jo Daviess Co., Illinois; CI = central Illinois; SC = South Carolina.
[b]1 = pooled standardization and Discriminant Function Analysis (DFA), 2 = independent standardization and DFA, and 3 = independent standardization and pooled DFA (see text).
[c]MasM = % males as males, MasF = % males as females, FasF = % females as females, FasM = % females as males.

Table 2. Size in mm of the distal end of the tibia of *Odocoileus virginianus* from Jo Daviess County (JD), Illinois, central Illinois (CI), Savannah River Plant, South Carolina (SC), and Brown Site (23VE3) (BR), Missouri. Standard deviations and sample sizes are enclosed in parenthesis. See text for definitions of actual and apparent measurements.

		Actual		Apparent	
Locality	Sex	Width	Depth	Width	Depth
JD	M	39.67 (1.20,23)	28.68 (1.23,23)	39.96 (0.92,22)	28.84 (1.07,22)
	F	36.74 (1.18,39)	26.43 (1.00,39)	36.65 (0.96,40)	26.40 (0.96,40)
CI	M	38.97 (1.99,14)	28.44 (1.75,14)	39.42 (1.43,14)	28.71 (1.46,14)
	F	36.42 (1.24,20)	26.14 (1.09,20)	36.11 (0.96,20)	25.96 (0.91,20)
SC	M	35.18 (1.56,26)	25.78 (1.21,26)	36.03 (0.97,19)	26.28 (0.95,19)
	F	32.95 (1.11,31)	24.01 (0.96,31)	32.94 (0.90,38)	24.09 (0.92,38)
BR	M	37.66[a]	27.63	38.20 (1.18,51)	27.95 (0.92,51)
	F	35.62	25.91	35.49 (1.07,72)	25.87 (0.88,72)

[a]Corrected values (see text).

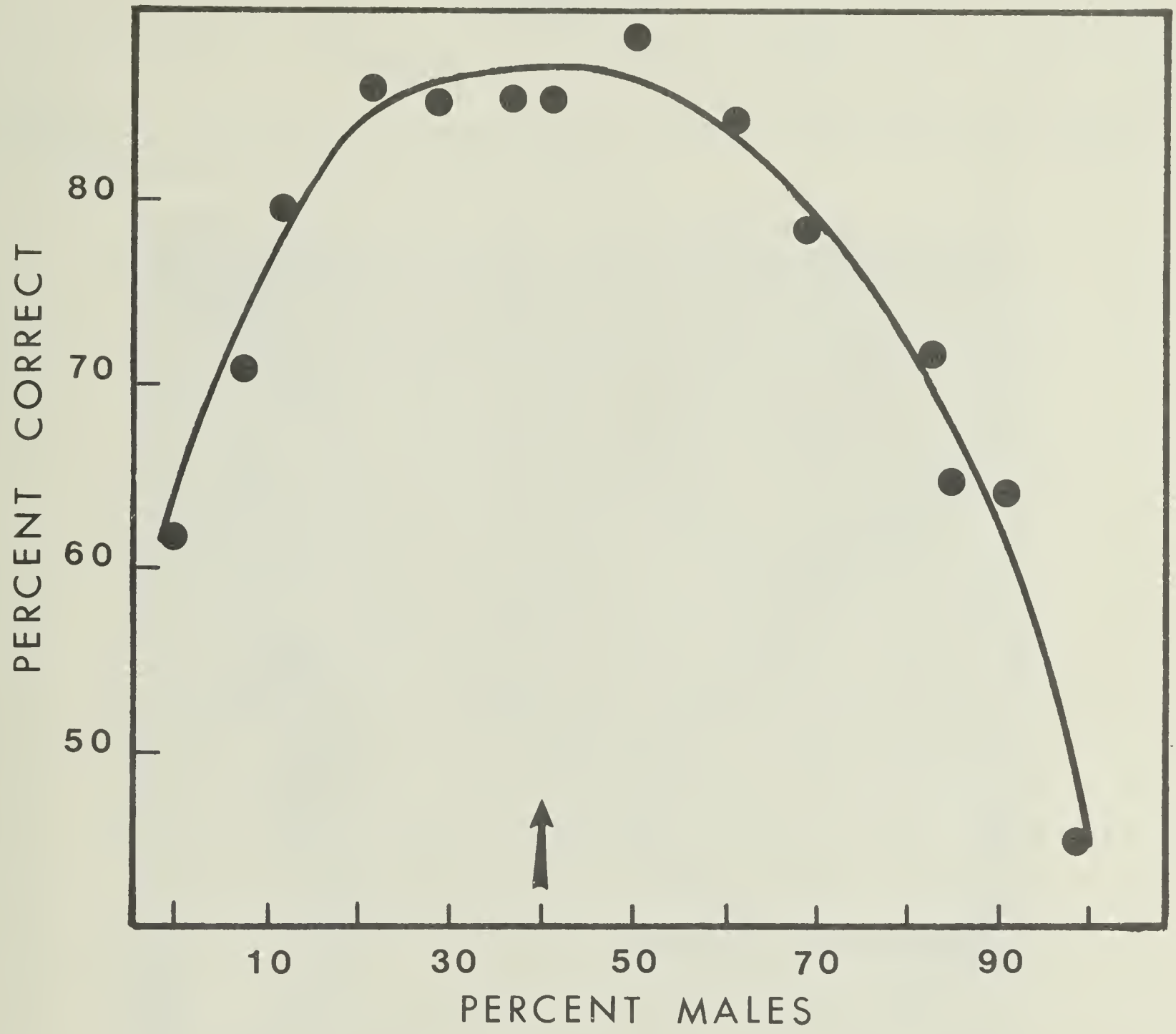

Fig.1. Effect of the sex ratio on classification using Option 3 statistical procedures (see text). Measurements of distal ends of tibias of deer from Jo Daviess County, Illinois, were treated as if they were from an archaeological site. The sex ratio was varied by random deletion and addition of specimens for 14 trial runs. Z-scores were recalculated for each run. The reference sample was comprised of deer from three localities: Jo Daviess County, central Illinois, and South Carolina. The curve is drawn by hand.

ERRATA

Volume 75, Numbers 3 and 4, 1982

WEIK, K.L. Asterocystis Smaragdina (Reinsch) Forti (Rhodophyceae): A New Algal Record for Illinois, pp. 175–177.

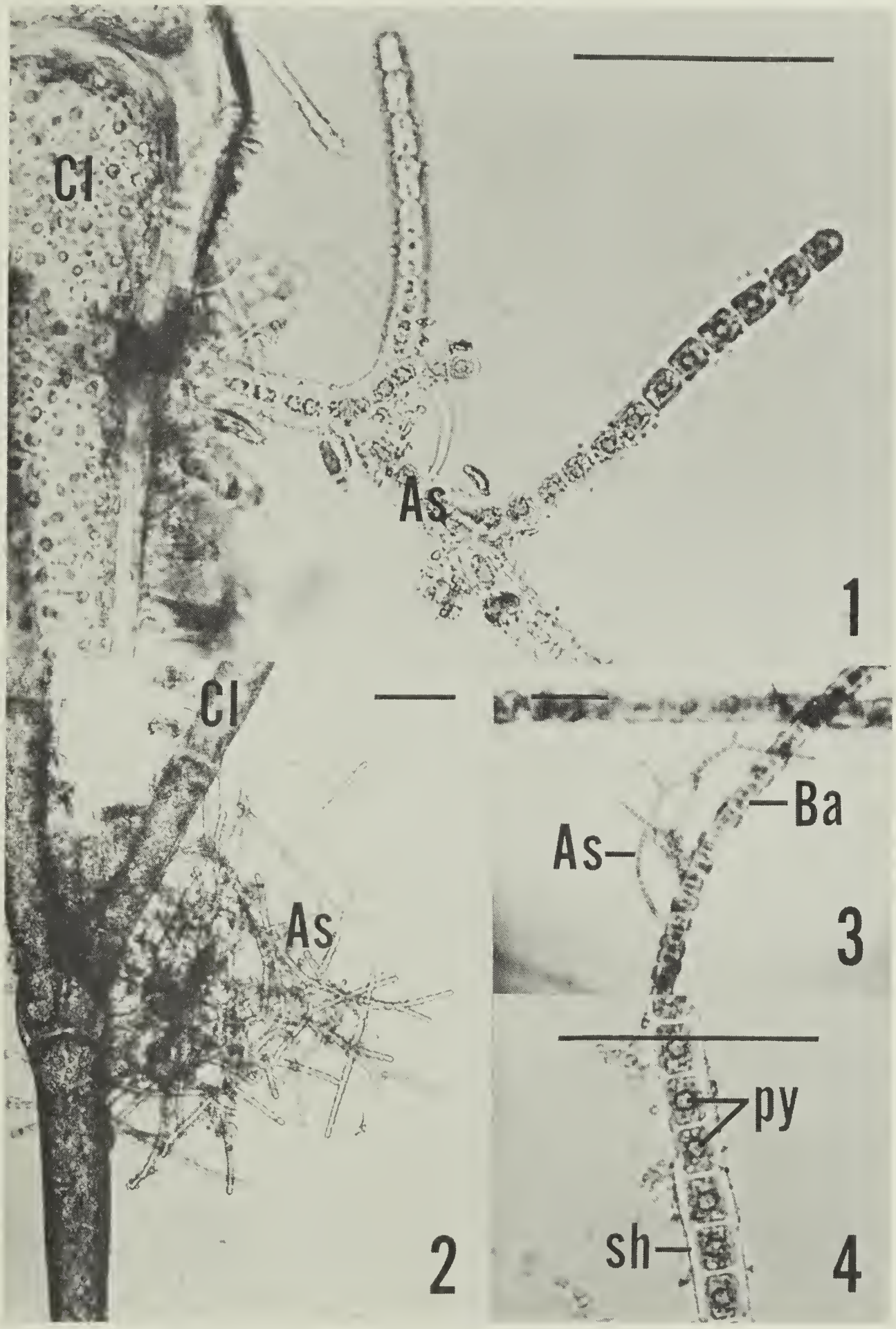

Fig. 1. Epiphytic habit of young thallus of *Asterocystis smaragdina* (As) on *Cladophora glomerata* (Cl). Note false-branching habit of epiphyte. Scale bar = 50 m.

Fig. 2. Older, repeatedly branched thallus on same host. Scale bar = 100 m.

Fig. 3. Young thallus attached to uniseriate filament of *Bangia atropurpurea* (Ba). Scale bar = 100 m.

Fig. 4. Short portion of a filament revealing the prominent, pyrenoid (py) in each cell. Note surrounding sheath (sh). Scale bar = 50 m.

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